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There are two known receptors for estrogens, ERα and ERβ. The existence of ERβ was only recently appreciated, and little is understood about its ability to be activated by intracellular signaling pathways in the absence of estrogens. The purpose of this research program is to characterize the ability of ERβ to by activated by various ligand-independent signaling pathways, and to characterize the structural regions of ERβ, in comparison to ERα, that regulate how this receptor isotype responds to intracellular cross-talk. We have found that stimulation of HeLa cells with forskolin and IBMX results in the activation of ERα and ERβ dependent expression in a receptor-dependent and promoter context-dependent manner, and that protein kinase A mediates this response. Factors that interact with an AP-1 binding site contribute to forskolin/IBMX activation of estrogen receptor-dependent gene expression, and do so in a manner that does not require the A/B domain of either receptor. At least c-Jun is able to stimulate ERα activity via the AP-1 binding site. Multiple coactivator proteins, predominantly of the steroid receptor coactivator (SRC) family and CREB binding protein (CBP) can stimulate ERα and ERβ activity induced by forskolin/IBMX pathways indicating that these coactivators can functionally interact with these receptors in the absence of ligand. Coactivation, however, does not appear to require SRC-1 phosphorylation as has been shown to be the case for progesterone receptors. This suggests that multiple pathways can be employed to regulate steroid receptor transcriptional pathways in a ligand-independent manner, and illustrates that understanding the mechanisms that control ERα and ERβ transcription activity provides insight into how transcription factor cross-talk can be regulated by a single agent.

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Introduction

Breast cancer remains the major cancer (excluding skin cancer) among women in the United States with more than 180,000 new cases anticipated in 2000. The requirement of estrogens for normal breast development is well documented (1). However, estrogens also have been linked to breast cancer, presumably through their ability to stimulate cell proliferation (2) and inhibition of estrogen action therefore has been a primary objective in the treatment, and more recently the prevention of, breast cancer. For many years, estrogen effects were thought to be mediated by a unique, high affinity intracellular receptor protein, the estrogen receptor (ER), that is a member of a superfamily of transcription factors (3,4). The basic mechanisms of ER activity have been ascertained. Hormone binding to ER results in receptor homodimerization and binding to specific enhancer DNA elements located in the promoter regions of target genes (5,6). This process, which is accompanied by increases in ER phosphorylation (7-12), enables "activated" receptors to regulate the transcription of hormone-responsive target genes and the resulting changes in mRNA and protein synthesis are ultimately responsible for alterations in cellular function. The structural features of the estrogen receptor (ERα) responsible for hormone binding, dimerization, DNA binding and transcriptional activation have been identified (3,13-16) and these studies have provided the basis of our understanding of the molecular mechanisms by which estrogens regulate the growth and differentiation of mammary tissues.

Clearly, the transcriptional activity of the ER can be regulated by estrogens, such as 17β-estradiol (E2). However, the ERα also can be activated in the absence of exogenous ligand by agents that stimulate intracellular signal transduction cascades (EGF, IGF-1, heregulin, dopamine, TPA and cAMP) (7,17-23) or inhibit protein phosphatases (okadaic acid) (19). Furthermore, cyclin D1, independent of cyclin-dependent kinases, also can activate the ER in the absence of estrogen (24). The ERα knock-out mouse model confirms that ERα is required for some but not all *in vivo* EGF effects and established the importance of ligand-independent activation of ER to physiological events (25). Most of these ligand-independent activation pathways (with the exception of cyclin D1) increase receptor phosphorylation (7,12,23,26,27) and mutation of the only known ligand-independent (EGF) phosphorylation site (serine^{118}) to an alanine residue abolishes EGF activation of the ER (28), suggesting that phosphorylation may play an important role in these activation pathways. However, this point mutant does not block cAMP-mediated gene expression and different domains are required to respond to EGF and cAMP signaling pathways (29), suggesting that multiple mechanisms must exist to enable ER to activate target gene expression in response to diverse regulatory events.

In 1996, a new member of the nuclear receptor superfamily was cloned from a prostate cDNA library (30). When the resulting cDNA was sequenced and expressed, it became apparent that a novel estrogen receptor had been identified. This new member of the nuclear receptor superfamily was named ERβ, and the original estrogen receptor was renamed ERα. The ERβ binds to estradiol with an affinity (K_d 0.4 nM) similar to ERα and binds to the same DNA response element as ERα (30-32). Thus, it is reasonable to predict that ERβ regulates the expression of at least a subset of ERα target genes. However, the relatively undeveloped mammary glands in the ERα knock-out mouse indicate that ERβ is not equivalent to ERα (33). The reasons for this are unclear, but could be related to differential expression and/or differences in the ability of α and β estrogen receptors to activate target gene expression. Mouse, rat and human ERβs are approximately 65 amino acids smaller than their corresponding α-receptors,

and the A/B, D and F domains (**Figure 1**) are poorly conserved (30,32,34). Furthermore, the ligand binding domains (domain E) of ERα and ERβ are only ~55% identical and rat ERα and ERβ receptors do not bind equally well to all ligands (31). The expression patterns of ERα and ERβ mRNAs are different but overlapping (31) suggesting that the genes for ERα and ERβ are independently regulated. However, both ERα and ERβ mRNA have been detected in human mammary gland, breast tumors and several human breast cancer cell lines (35,36). Taken together, these data suggest that ERβ is likely to play a role in mediating estrogen action in mammary gland, but that this receptor is unlikely to be functionally equivalent to ERα.

The identification of a second estrogen receptor raised a number of important biological questions such as, what is the expression of ERβ, relative to ERα, in normal and malignant mammary tissue? However, knowledge regarding the expression of ERβ in mammary gland will be of limited value without detailed information on the transcriptional activity of ERβ. Do ERα and ERβ respond similarly to ligand-independent pathways? Are currently used antiestrogens equally effective antagonists of ERα and ERβ? Do ERα and ERβ activate the same target genes to a comparable extent? Studies of this nature will provide the information necessary to determine whether resources are required to develop new strategies to more effectively and/or selectively block ERα- and ERβ-mediated estrogen effects. Indeed, increasing evidence demonstrates that ERα and ERβ are not functionally equivalent, and our experiments reported below contribute to the foundation upon which new strategies to regulate ERα and ERβ biological activity can be developed. Moreover, comparing and contrasting the structure/function relationships of ERα and ERβ with respect to activation by ligand-independent pathways represents a novel approach to study mechanistic questions relating to activation of gene expression in the absence of estrogens.

Body

A new member of the nuclear receptor superfamily, ERβ, has been identified that binds to estrogens with high affinity, and binds to the same DNA response elements as the classical estrogen receptor, ERα. Both of these ligand-regulatable transcription factors possess a well-defined, centrally located, DNA binding domain and carboxy-terminal domain, which contains a ligand-dependent activation function (AF-2); however the amino terminus which possesses a second activation function (AF-1) is poorly conserved. Thus, it is highly likely that that the biological activity of ERβ will differ from that of ERα. This hypothesis is being tested in the following two technical objectives:

1. To determine if estrogen-independent signaling pathways can stimulate ERβ transcriptional activity.
2. To determine what regions of ERβ contribute to its estrogen-independent transcriptional activity and to compare these regions to known ERα activation functions to characterize the structural features of these receptors that contribute to their respective biological properties.

The originally reported form of ERβ represented a truncated version of the subsequently identified full length form of the receptor. In the first year of this award, we conducted experiments directed towards resolving differences in activity and expression of the full-length

and truncated forms of ERβ. All of our studies in years 2 and 3 utilized the full-length form of ERβ, (unless deletion mutations were being analyzed).

In the second year, we continued our analyses of the ability of ERβ to be activated by ligand-independent, cAMP-stimulated, signaling pathways. Both ERα and ERβ are activated in cells treated with forskolin and isobutylmethylxanthine (IBMX). Forskolin is an activator of adenylyl cyclase and IBMX is a phosphodiesterase inhibitor, and treatment of cells with these compounds therefore results in an increase in intracellular cAMP levels. In transient transfection assays, 10 μM forskolin and 100 μM IBMX stimulated the ERβ activation of ERE-Elb-CAT target gene by ~6-fold while ERα-dependent gene expression was stimulated by ~3-fold. Minimal change in target gene expression was observed in cells transfected with the reporter gene and an empty expression vector indicating that the increased CAT activity is receptor-dependent. Furthermore, an ERα mutant possessing point mutations in its DNA binding domain (C201H/C205H) was unable to mediate forskolin/IBMX-induced CAT gene expression indicating that receptor binding to DNA was required. Further analysis demonstrates that an target gene lacking the ERE cannot be stimulated by the forskolin/IBMX-induced signaling pathway in cells expressing ERα or ERβ. Thus, intracellular cAMP signaling pathways have the potential to activate the transcriptional activity of both ERα and ERβ, and this activation is dependent on the expression of an estrogen receptor, the receptor's ability to bind to DNA and the presence of an estrogen response element within the target gene. To ensure that target gene expression resulted from forskolin/IBMX activation of the cAMP-dependent/protein kinase A (PKA) signaling pathway and not a non-specific event, we demonstrated that the specific protein kinase inhibitor, H89, blocked forskolin activation of both receptor isotypes, but not transcriptional activity stimulated by E2, supporting the hypothesis that activation occurs via a cAMP/PKA dependent signaling pathway.

The preceding experiments were performed with the ERE-E1b-CAT or ERE-tk-CAT target genes which consist of an estrogen response element linked to a TATA box or thymidine kinase promotor, and the CAT reporter gene. To investigate whether the target gene influenced the ability of forskolin/IBMX to activate receptor-dependent gene expression, the same experiment was repeated, but using other target genes. As we also demonstrated previously, the expression of the pS2-CAT, pATC0, pATC1, pATC2, ERE-E1b-Luc and pC3-Luc target genes was not stimulated by forskolin/IBMX, although E2 increased gene expression in every target gene that possessed an ERE. In contrast, the activity of the ERE-tk-CAT, ERE-Elb-CAT and pC3110-tk-Luc target genes was increased. In year 3, we also examined forskolin/IBMX induction of the oxytocin promoter and found that the cAMP pathway stimulated ERα but not ERβ transcriptional activity. This indicates that the ability of the cAMP-dependent PKA pathway to activate target gene expression was dependent on the nature of the reporter gene examined. The majority of these target genes contain consensus EREs, so we therefore turned our attention to other potential transcription factor binding sites that are present within the synthetic target gene vectors, in order to determine what role, if any, they play in forskolin/IBMX activation of ER-dependent gene expression.

Many vectors have an imperfect AP-1 binding site (also known as a TPA responsive element or TRE) located several hundred base pairs upstream of their minimal promoters. Both the ERE-E1b-CAT and ERE-tk-CAT vectors have such a site. In order to determine if this binding site contributed to the overall activation of gene expression following forskolin/IBMX stimulation of

cells, we made a four nucleotide insertion within the putative TRE of ERE-Elb-CAT that prevents AP-1 from binding to DNA (37). Interestingly, forskolin/IBMX was unable to activate ERβ-dependent expression of the resulting mutated target gene even though E2 could still stimulate ERβ activity. In contrast, mutation of the putative AP-1 site did not block forskolin/IBMX activation of target gene activity by ERα, although it decreased the relative magnitude of the response. Similar results were obtained when the AP-1 site was removed through a more extensive deletion of 195 bp surrounding the AP-1 binding site. These experiments suggested that AP-1 sites were contributing to the ability of ERα and ERβ to stimulate ERE-dependent gene expression, and that factors that bound to the TRE and ERE sites were cooperating to bring about activation of transcription. In support of this, we and others (38,39) have shown that forskolin/IBMX activates AP-1-dependent gene expression. Although the reporter genes that we have used in our studies are by their very nature, synthetic in origin, it is interesting to note that TREs are widely distributed in the promoter region of many endogenous genes, including the progesterone receptor which has been shown to be stimulated in an ER-dependent manner by treatment of cells with IBMX and cholera toxin (40), an agent that like forskolin/IBMX will stimulate intracellular cAMP production/accumulation and activation of a PKA signaling pathway, or forskolin (41).

It has been reported previously that ERα can interact with c-Jun, one component of the AP-1 transcription factor directly through the receptor's A/B domain (42). However, both ERα and c-Jun are also able to bind to coactivators, such as CBP/p300 (43-45), and it is possible that the cooperative functional interactions between these two transcription factors are direct (*e.g.* they bind to one another) or indirect (*e.g.* they interact via association with a common coativator). To begin to distinguish between these two possibilities, we analyzed the ability of ERα and ERβ deletion mutants lacking their A/B domains to be activated by the forskolin/IBMX-stimulated signal transduction pathway. In our first experiment, the A/B domains of ERα and ERβ were deleted to generate expression vectors for ERα-179C and ERβ-143C, respectively. These deletion mutants were tested for their ability to activate the expression of the ERE-Elb-CAT target gene in response to the forskolin/IBMX-induced signaling pathway. Forskolin/IBMX activated the transcriptional activity of ERα-179C and ERβ-143C, the former to an extent reduced in comparison to its respective wild type receptor. However, when assays were performed with the mutated target gene, ERE-E1b-CAT (mTRE) in which the TRE has been disrupted by a four nucleotide insertion within the putative AP-1 site, neither receptor deletion mutant was able to stimulate transcription of the target gene. Taken together, this indicates that activation of target gene transcription by the cAMP-dependent/PKA signal transduction pathway requires an estrogen receptor as well as another activity dependent on the TRE site, and that these interactions do not require the A/B domain of either ERα or ERβ.

Our experiments in the third year continued to examine the ability of cAMP signaling pathways to stimulate the activity of ERα and ERβ. Because our studies in year 2 demonstrated that the amino-terminus of ERα contributed to this response (see above), and because phosphorylation of this region of ERα contributes to AF-1 activity, we examined whether mutation of the amino-terminal phosphorylation sites affected the ability of forskolin/IBMX to stimulate gene expression. When three of the major ERα amino-terminal phosphorylation sites are mutated to alanine residues, the ability of forskolin/IBMX to stimulate transcription, relative to E2-induced gene expression is maintained. It was apparent, however, that these mutations decreased the overall ability of estradiol or forskolin/IBMX to stimulate activity; the former

result was expected from previous findings (10,46). This indicated that these phosphorylation sites were not specifically required for activation of ERα transcriptional activity by this pathway.

We have concentrated a significant amount of effort examining the ability of proteins to facilitate the interaction between ERs and the factors that bind to the TRE site. Our assumption in our year 2 studies had been that the factor that binds to the TRE site was AP-1. AP-1 is composed of either homo- or heterodimers within the Jun family (c-Jun, JunB and JunD) or between heterodimers of the Jun and Fos (c-Fos, FosB, Fra1 and Fra2) families (47). We therefore examined whether AP-1 proteins could functionally interact through the TRE site. HeLa cells were transfected with an ERα expression vector and a reporter gene with (TRE-ERE) or without (Δ*Nde-Eco*-ERE) the TRE site. In addition, these cells were transfected with expression vectors for either c-Jun, c-Fos or combinations of the two. Our data demonstrate that c-Jun is able to significantly increase CAT gene expression only when a TRE site was present in the vector. c-Fos on its own had little effect on the magnitude of gene expression, and had little effect on c-Jun activity. Thus, the AP-1 protein c-Jun can stimulate ERα activity only when a binding site (TRE) for this protein is present. Moreover, this result demonstrates that AP-1 proteins are able to exert the TRE-dependent effects on ERα activity that we have observed.

In the past year, to further investigate the role of AP-1 proteins in cAMP activation of ER target gene expression, we first determined whether forskolin/IBMX treatment increased activation of target gene expression by increasing levels of c-Jun or Fos family members in treated cells. By Western blot analysis, neither Jun or Fos expression levels were increased in forskolin/IBMX treated cells, indicating that an increase in activity was not due to overexpression of either of these transcription factors (**Figure 2**). Indeed by Western blot with using antibodies from several different manufacturer's (Cell Signaling, Oncogene and Upstate Biotechnology) endogenous c-Jun expression was undetectable. Transient transfection of an expression vector for c-Jun was therefore used as a positive control in these experiments. It is possible that forskolin/IBMX could increase activity via increasing the DNA binding activity of existing AP-1 proteins to the TRE site in the target gene promoter. To test this possibility, electrophoretic mobility band shift assays were performed. As shown in **Figure 3**, forskolin/IBMX does not increase the amount of material bound to the oligonucleotides encompassing the TRE site. Moreover, antibodies to both Fos and c-Jun reduce the material bound to the TRE site, indicating that these proteins do indeed interact with this site. This was important to demonstrate since the putative TRE site in our reporter construct is not a consensus TRE site. Finally, electrophoretic mobility band shift assays performed with the mutated TRE oligonucleotides that correspond the mTRE sequences used in the reporter construct mentioned above do not show significant binding to either c-Jun or fos, consistent with the inability of this sequence to mediate forskolin/IBMX induction of target gene expression.

Finally we in the last year have begun to address the issue of whether AP-1 proteins are important for cAMP induction of endogenous target genes in breast cancer cells using MCF-7 cells as our model. For these studies we have established a collaboration with Dr. Powel Brown in which we are using a dominant negative c-Jun construct generated and characterized by Dr. Brown (48,49). These dominant negatives are based on deletion mutants of c-Jun in which the activation domain of this transcription factor is deleted. The basic dominant negative (called Tam67) utilizes only the transactivation domain deletion, while a more severe mutant, called Tam67/Fos consists of deletion of the transactivation domain as well as mutation of the dimerization domain that blocks heterodimerization with Fos. In our studies, transfection of

Tam67 or Tam67/Fos inhibits activation of the ERE-E1b-CAT reporter gene by forskolin, and demonstrates that these dominant negatives should be suitable for determining whether blocking AP-1 activity will block expression of endogenous target genes in response to cAMP stimulation (**Figure 4**). We have therefore obtained from Dr. Brown's laboratory MCF-7 cell lines (48) stably transfected with a dominant negative c-Jun (both the Tam67 and Tam67/Fos versions). The dominant negative protein in under the control of the Tet regulated system such that in the presence of doxycycline the Jun proteins are not expressed. Upon withdrawal of the doxycycline, the mutant Jun proteins are expressed as shown by Western blot (**Figure 5**). This was initially characterized with an antibody directed toward the Flag epitope that with which the dominant negative proteins were tagged. Subsequently we demonstrated that the Tam67 dominant negative protein is expressed at levels greater than the endogenous Jun (**Figure 6**) suggesting that this cell line will be suitable to test whether block endogenous c-Jun activity will block cAMP induction of ER target gene expression. We are currently examining whether the dominant negative Jun will block cAMP induction of known responsive target genes (*e.g.* pS2, progesterone receptor, cathepsin D) in MCF-7 cells; these studies are ongoing.

Since c-Jun is known to bind to ERα via its amino-terminus, and since this domain is dispensable for cAMP activation as long as there is a TRE site in the vector, we hypothesized that another molecule must act as a bridging factor between these two transcription factors. This putative factor would need to be able to interact with both c-Jun as well as ERs. Furthermore, this putative factor would need to functionally interact with both ERα and ERβ through the carboxy-terminal portion of either receptor. We have now evaluated a number of candidates. The first is a coactivator of c-Jun and steroid receptors called JAB1 (50). When JAB1 was cotransfected into cells, it very modestly enhanced the activity of both ERα and ERβ stimulated by either forskolin/IBMX or E2 regardless of whether a TRE site was present in the promoter. Due to the relative lack of JAB1 activity in this system, we did not pursue this further. We also considered the activity of cyclin D1, a protein which has been implicated in the activation of ER by virtue of its ability to promote SRC-1 and P/CAF recruitment to the receptor (51,52). In addition, it had been recently shown that cAMP treatment of cells enhanced the interaction between ERα and cyclin D1 (53). Surprisingly, we found that coexpression of cyclin D1 in our cells resulted in a decrease in ERα-dependent gene expression, contrasting with the results of others. We have repeated this experiment multiple times and the result is consistent. It is possible that differences in cellular environment may contribute to these differences. However, since cyclin D1 overexpression was not able to enhance ERα and ERβ transcriptional activity stimulated by forskolin, this factor seems unlikely to be a contributing factor facilitating cAMP interactions between AP-1 and ERs.

One final approach that we have employed is to develop a dominant negative form of CBP. CBP is a coactivator for both c-Jun (45) and ERα (54). We made use of the fact that the region of CBP required for interaction with c-Jun (amino acids 461 to 661) is distinct from the residues required to interact with ERα (amino acids 1-101) (55). Thus, by creating an expression vector for the c-Jun interaction region (JIR) one would expect to block the activity of CBP to interact with c-Jun, but not with ER. When the JIR fragment was overexpressed in cells, we observed a decrease in ERα and ERβ activity both stimulated by E2 as well as by forskolin/IBMX pathways. On an ERE-E1b-CAT reporter, this would be expected since as we showed in figure 4, the TRE site contributes to E2 and forskolin/IBMX activity.

We had previously shown that SRC-1 overexpression could coactivate ERα activity stimulated by SRC-1 overexpression (56). To confirm and extend this observation, we examined the ability of SRC family and the CBP coactivators to stimulate that transcriptional activity of ERα and ERβ. All four coactivators stimulated the activity of both receptors whether activated by either E2 or forskolin/IBMX. Moreover, this coactivation could be observed in the absence of the TRE site, indicating that interaction with ER alone is sufficient to allow the coactivators to stimulate ligand-independent (cAMP) ER-dependent gene expression (data not shown). It has been recently published that cAMP treatment of cells results in phosphorylation of the SRC-1 coactivator, and that this phosphorylation is associated with a increase in the ability of coactivator to stimulate the activity of the chicken progesterone receptor stimulated with either progesterone or 8Br-cAMP, another agent capable of initiating cAMP signaling pathways (*e.g.* activation of PKA) in cells (57). We therefore examined the ability of SRC-1 phosphorylation mutants to stimulate gene expression induced by treatment of cells with forskolin/IBMX. Mutation of the SRC-1 phosphorylation sites does not block cAMP activation of ER-dependent gene expression. This is important because it indicates that cAMP activation of the transcriptional activity of different members of the steroid receptor superfamily (*e.g.* ER and PR in this case) may be achieved by multiple mechanisms, and that various components of the steroid receptor gene expression pathway can be targeted for cAMP regulation. This also sheds light on how it is possible that activation of ERα and ERβ may be distinct.

We also initiated studies in year 3 to examine the ability of the dopamine pathway, which has been shown to ligand-independently activate ERα (17), to activate ERβ transcriptional activity. For these studies we used a synthetic full dopamine receptor agonist, SKF-82958 (±-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrehydro-1H-3-benzazepine, since this compound is more stable than dopamine. As previously reported (58), SKF-82958, like dopamine, stimulated ERα transcriptional activity, and this was inhibited by the pure ER antagonist ICI 182,780. We then examined activation of human ERβ using the ERE-E1b-Luc reporter gene. SKF-82958 was not able to significantly activate ERβ-dependent gene expression in comparison to the ability of this compound to stimulate ERα transcriptional activity. SKF therefore appears to preferentially activate ERα. To ensure that SKF-82958 induction of ERα-dependent gene expression was not due to ligand stabilization of ERα expression, Western blot analysis of ERα expression in cells treated with vehicle, E2 and SKF-82958 was performed, and like E2 and dopamine (17,59), SKF was found to down-regulate the expression of ERα in HeLa cells. Dose response studies indicated that half-maximal induction of ERα-directed gene expression by SKF-82958 occurred at 2 μM (data not shown). In contrast, maximal dopamine induction of ER-directed gene expression occurs at 100-250 μM (17,19,28), suggesting that SKF-82958 is a more potent activator of this response. However, the potency (K_m) and maximum efficacy of SKF-82958 induction of cAMP are similar to that for dopamine in rat brain striatum after treatment *in vivo* (60). This discrepancy suggested that there may be mechanistic differences in the ability of SKF-82958 and dopamine to stimulate ERα transcriptional activity.

To investigate this further, SKF stimulation of cAMP production in HeLa cells was examined by RIA and compared to the ability of SKF to activate ER-dependent gene expression. No correlation was found, as micromolar doses of SKF-82958 failed to significantly elevate cAMP levels. To more closely mimic conditions under which our transactivation assays are performed, the ability of SKF-82958 to stimulate cAMP response element (CRE)-dependent transcription

was assessed. The -169αCG-CAT gene is composed of a fragment of the human chorionic gonadotropin gene promoter containing a CRE element, linked upstream of the CAT reporter gene and is activated by cAMP stimulation of the CREB transcription factor (61). The -100αCG-CAT reporter gene which lacks the CRE was used as a negative control. CRE-dependent transcription was stimulated by 8Br-cAMP and, more modestly, by dopamine. However, there was no stimulation of CRE-dependent transcription by E2 or SKF-82958. These results suggest that SKF-82958 is not acting through stimulation of a cAMP-dependent dopaminergic signaling in this system. This result led to a consideration of whether this compound activated ER-dependent gene expression through direct binding to ERα. This question is further underscored by the ring structure of this synthetic D1 receptor agonist which is reminiscent of the structures of some ER agonists and antagonists (62).

In order to determine whether SKF-82958 could bind to ERs, whole cell competitive hormone binding assays were performed in HeLa cells transfected with expression vectors for either ERα or ERβ. Cells were incubated with [^{3}H]estradiol and increasing amounts of unlabeled E2, SKF-82958 or dopamine. The displacement curves for ERα and ERβ indicate that SKF-82958 can compete weakly with estradiol for binding to both forms of ER but that dopamine is unable to do so. The average relative binding affinities of SKF-82958 in comparison to E2 (100) for ERα (0.077 ± 0.018; n=4) and ERβ (0.069 ± 0.009; n=3) are similar and are comparable to those measured by other investigators for low affinity ER agonists such as bisphenol A (31). This result suggests that activation of ERα-dependent gene expression may arise through SKF-82958 binding to ERs and serving as a weak receptor agonist. Therefore, instead of SKF ligand-independent activation of ERα and not ERβ, it appears that SKF bind to both ERs and acts as a ERα subtype selective agonist. The identification of these types of compounds are important because the contribute to the ongoing identification and utilization of subtype selective ligands that will enable ERα and ERβ specific functions to be investigated.

Lastly, in part, because the SKF-82958 experiments revealed that small molecules that have differential effects on ERα and ERβ transcriptional activity an be acting as agonists, we have also established a collaboration with Drs. Austin Cooney and Fernando Larrea. We have examined the ability of some A-ring reduced metabolites of 19-nor synthetic progestins (norethindrone and Gestodene) to regulate ERα and ERβ activity. We found that the 3β,5α derivatives of these compounds have a preferential ability to bind to as well as activate ERα *versus* ERβ. This indicates that not only are there differences in how ligand-independent pathways regulate ER transcriptional activity, but also how ligands interact with and stimulate the activity of ERs.

Key Research Accomplishments for Past Year

1. Treatment of cells with forskolin/IBMX does not increase cellular expression of c-Jun or Fos family members.
2. Both fos and c-Jun bind to the putative TRE sequence that confers cAMP responsiveness in target gene expression.

3. Treatment of cells with forskolin/IBMX does not increase the DNA binding activity of c-Jun or Fos family members.
4. Dominant negative c-Jun inhibits cAMP induction of ER target gene expression in *trans*-activation assays.

Reportable Outcomes for Entire Project Duration

1. Larrea F, García-Becerra R, Lemus AE, García GA, Grillasca I, Pérez-Palacios G, Jackson K, Smith CL and Cooney AJ (2001): A-ring reduced metabolites of 19-nor synthetic progestins as selective agonists for estrogen receptor-α. *Endocrinology* 142:3791-3799.
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3. Walters MR, Dutertre M and Smith CL (2002): SKF-82958 is a Subtype-Selective Estrogen Receptor-α (ERα) Agonist that Induces Functional Interactions between ERα and AP-1. *Journal of Biological Chemistry* 277:1669-1679.
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6. Coleman KM, Lam VD, Lanz RB, O'Malley BW and Smith CL: Stimulation of Estrogen Receptor α and β Transcriptional Activity by the RNA Coactivator, SRA. Keystone Symposia – Nuclear Receptor Superfamily. Steamboat Springs, CO March 25-31, 2000.
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Copies of items 3, 4, 8 and 9 can be found in the Appendix. Copies of items 1, 2 and 5-7 were submitted in previous year's reports.

Conclusions

The originally published amino acid sequence of ERβ represents an amino-terminally truncated form, which lacks the first 45 amino acids of this receptor subtype. In transient transfection assays, ERα is clearly more active than the long and short forms of ERβ. The potentially large differences in $ER\beta_S$ and $ER\beta_L$ expression levels indicate that their relative expression levels must be taken into account when considering transactivation activity. Furthermore, the AF-1 activity of ERα exceeds that of ERβ and this likely contributes to the relative differences in transcriptional activity observed for these two receptor isotypes.

Both isotypes of estrogen receptor (α and β) can be activated in the absence of exogenous estrogens. In cells treated with forskolin and IBMX, ERα and were activated by a cAMP signaling pathway. This indicates that there is sufficient homology between these two receptor isotypes to mediate activation of gene expression by this signaling pathway. This activation pathway required the expression of estrogen receptors within the target cell, the presence of an estrogen response element in the target gene, and that the receptor can bind to DNA. However, the stimulation of receptor-dependent transcription can be significantly enhanced by the presence of the binding site for another transcription factor, in these studies an putative AP-1 binding site. Furthermore, the ability of ERα and ERβ to participate in this combinatorial response differs, supporting our original hypothesis that the ability of both estrogen receptor isotypes needs to be examined in order to determine the potential of each of these receptors to respond to ligand-independent signaling pathways.

We have investigated the ability of a number of coactivator proteins to stimulate ERα and ERβ transcriptional activity, and found that their potential to do so is variable. All SRC family members can coactivate ERα and ERβ transcriptional activity stimulated by forskolin/IBMX. This is consistent with their ability to interact with the receptor carboxy-termini, and the previously reported requirement of this domain for cAMP activation of transcription. In addition, CBP can also coactivate both forms of ER. These molecules are therefore are prime candidates to examine the relationship between ER and c-Jun with respect to cAMP activation of gene expression. Intriguingly, cAMP activation of PR and ERα also appears to be quite distinct. The former is not phosphorylated in response to cAMP signaling and appears to rely on modification to SRC-1. In contrast, ERα is phosphorylated and does not required SRC-1 phosphorylation to being about cAMP activation of gene expression. Thus, this pathway can communicate to different members of the steroid receptor superfamily via distinct signaling pathways. This is important because it implies that inhibition of cAMP activation of steroid receptor transcriptional activity may need to inhibit multiple molecule events to be achieved.

As anticipated, the experiments performed to date have provided information on the transcriptional activity of ERβ relative to ERα, as well as the ability of ERβ to respond to an alternative signaling pathway, induced by elevated intracellular cAMP, in the absence of estrogens. Taken together, this information will increase our understanding of the molecular mechanisms by which ERα and ERβ respond to cross-talk pathways within a cell. It also will provide a framework for critical evaluation of whether it is possible to selectively regulate ERα and ERβ transcriptional activity.

Personnel Receiving Salary Support from this Award

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Appendices

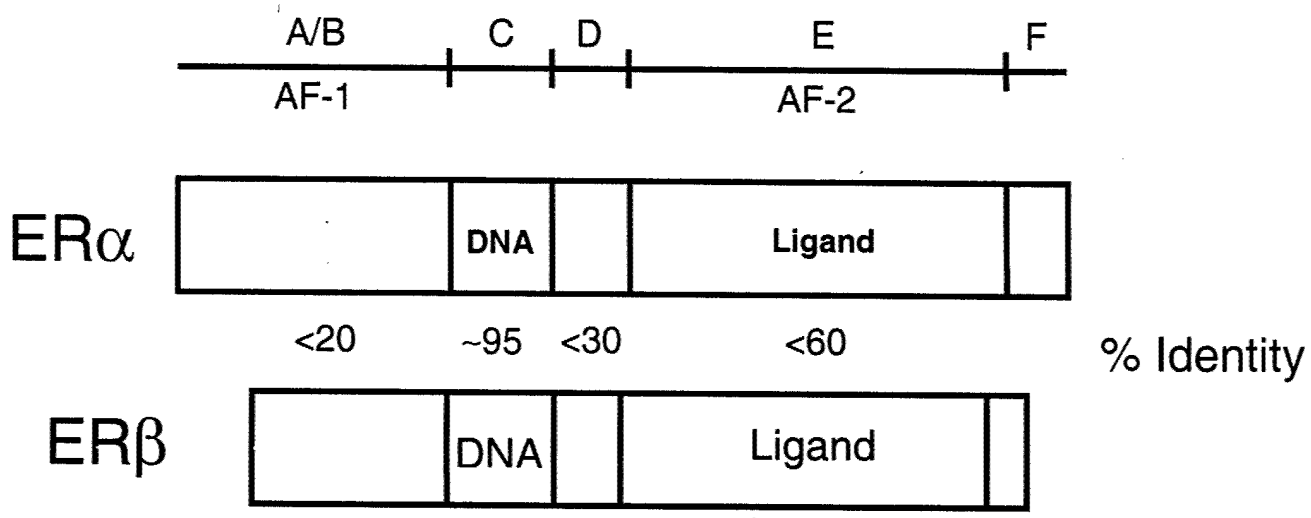

Figure 1: Structure of ERα and ERβ. The *top panel* represents the location of various regions of estrogen receptor (A to F) and its activation functions (AF-1 and AF-2). The bottom panel represents the comparative structure of ERα and ERβ. Values given between the two receptor forms represent approximately homologies in their respective amino acid sequences.

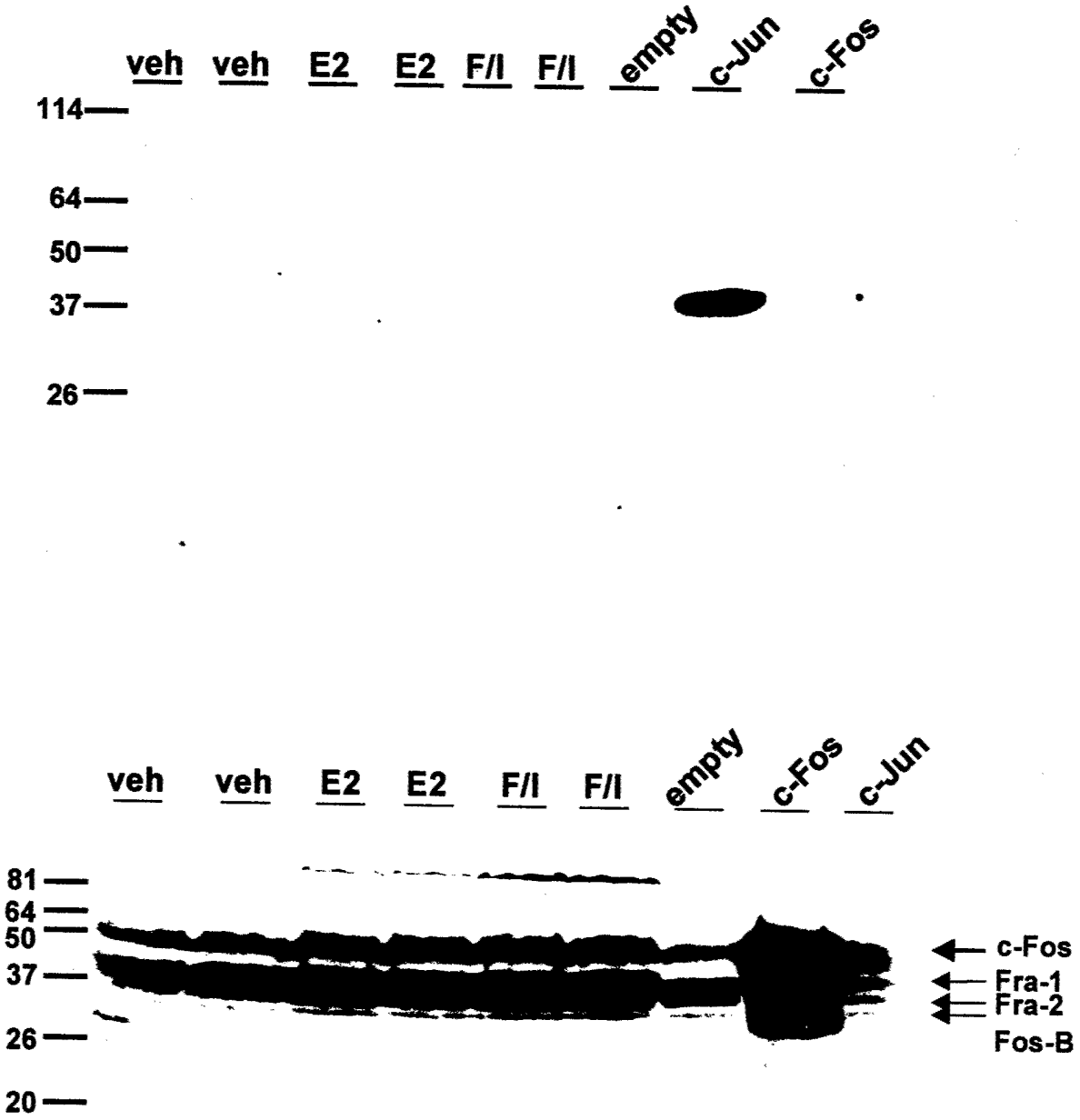

Figure 2: Western blot analysis of c-Jun and Fos expression. HeLa cells were treated for 24 hours with EtOH (vehicle), 1 nM estradiol (E2) or 10 μM forskolin and 100 μM IBMX (F/I). Cell extracts were prepared, resolved by SDS-PAGE, transferred to nitrocellulose and assessed by Western blot analysis for c-Jun using a Cell Signaling antibody (*top*) or Fos family members using a Santa Cruz antibody (*bottom*). .

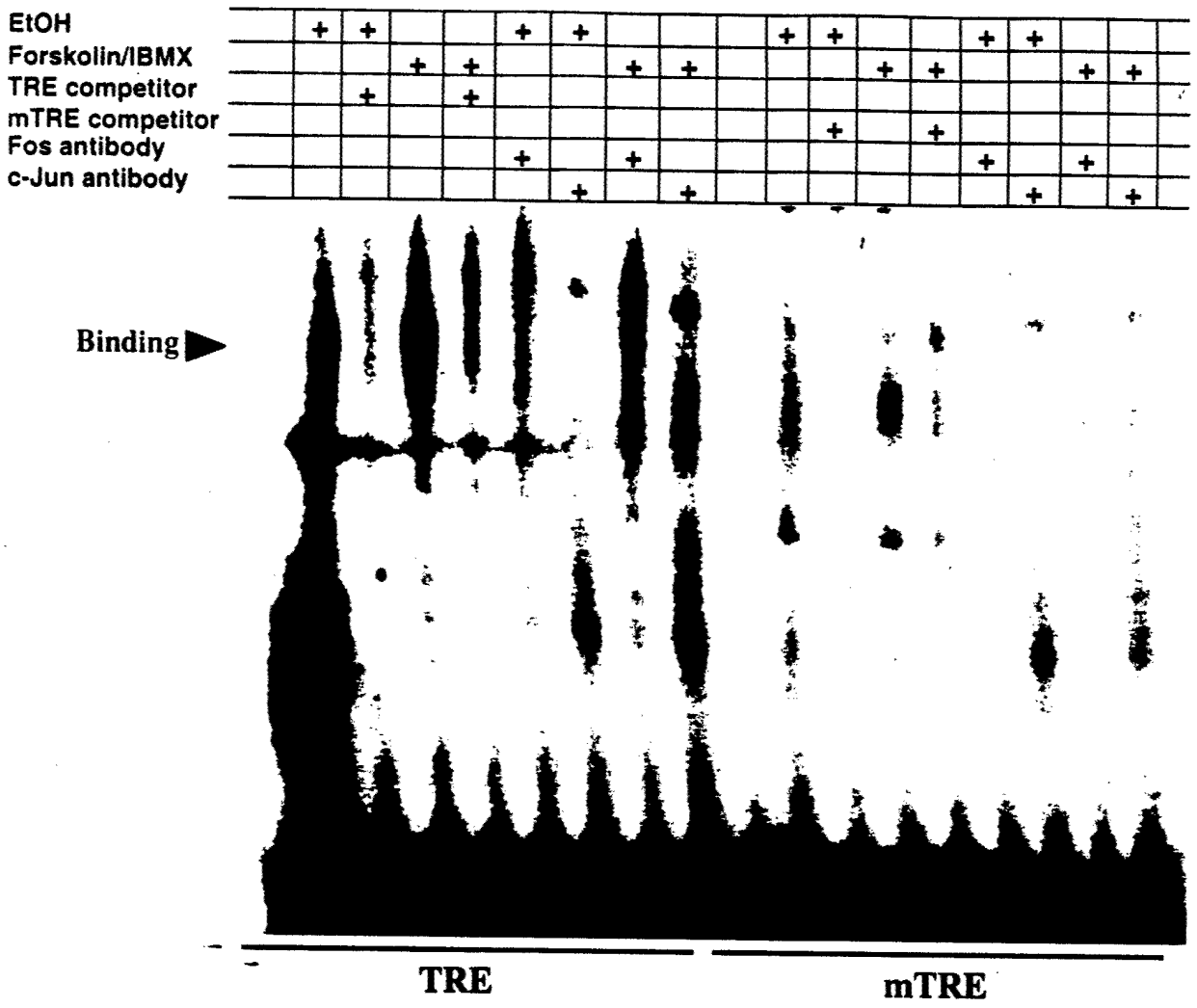

Figure 3: Electrophoretic mobility shift assay of cellular factors binding to the TRE site. A c-Jun/Fos complex can bind to the ^{32}P-labeled TRE probe in the absence and presence of forskolin/IBMX (*left side*). The sequence of the TRE probe was derived from the putative TRE site located in the ERE-E1b-CAT target gene. As expected, very little binding occurs with the mutated TRE (mTRE; *right side*).

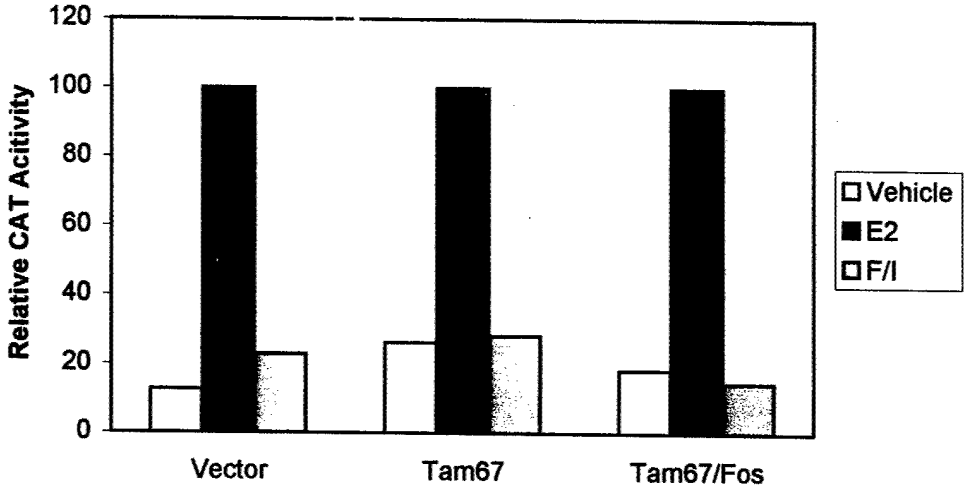

Figure 4: Overexpression of dominant negative c-Jun inhibits ERα transcriptional activity. HeLa cells were co-transfected with expression plasmid for dominant negative c-Jun (Tam67 or Tam67/Fos) or corresponding empty vector along with an ERα expression vector and ERE-E1b-CAT reporter gene. Total DNA levels were normalized in each group by co-transfecting appropriate levels of the empty plasmid. Cells were treated with ethanol (vehicle), 1 nM E2 or 10 μM forskolin/100 μM IBMX. Bars represent values expressed relative to the CAT activity (100) induced by E2 treatment.

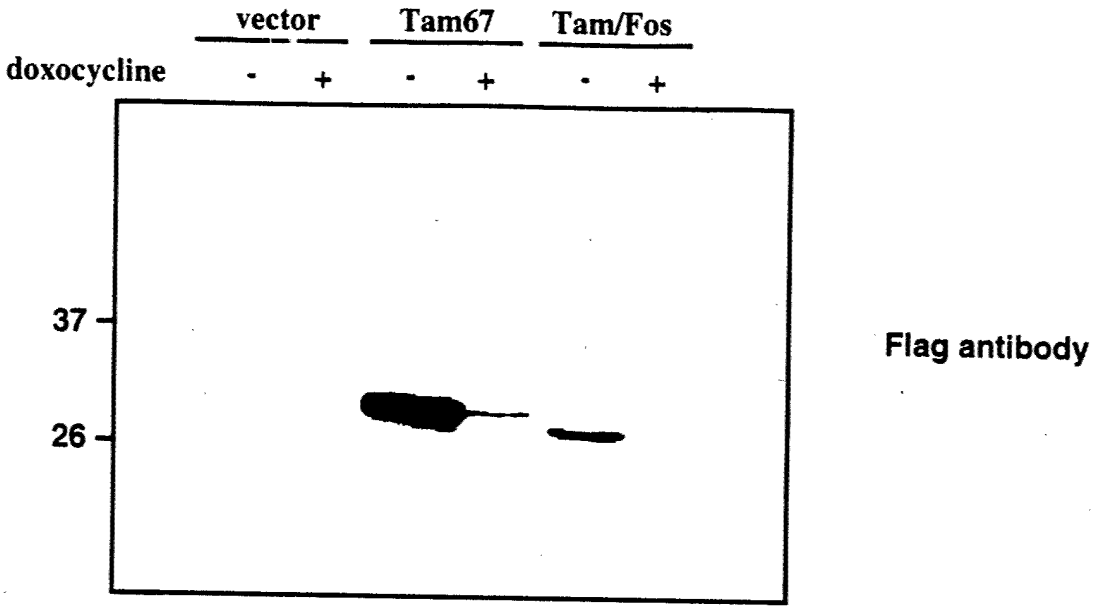

Figure 5: Induction of Tam67 and Tam67/Fos expression in MCF-7 cells. MCF-7 cells stably transfected with Tet-regulated expression vectors for vector alone (vector), Flag-tagged Tam67 or Flag-tagged Tam67/Fos were cultured in the absence ('-') or presence ('+') of doxycycline for 6 days. Cell extracts were prepared, resolved by SDS-PAGE, transferred to nitrocellulose and assess for dominant-negative c-Jun expression by Western blot with an antibody to the Flag epitope. This figure shows induction of dominant negative c-Jun expression upon withdrawal of doxycycline.

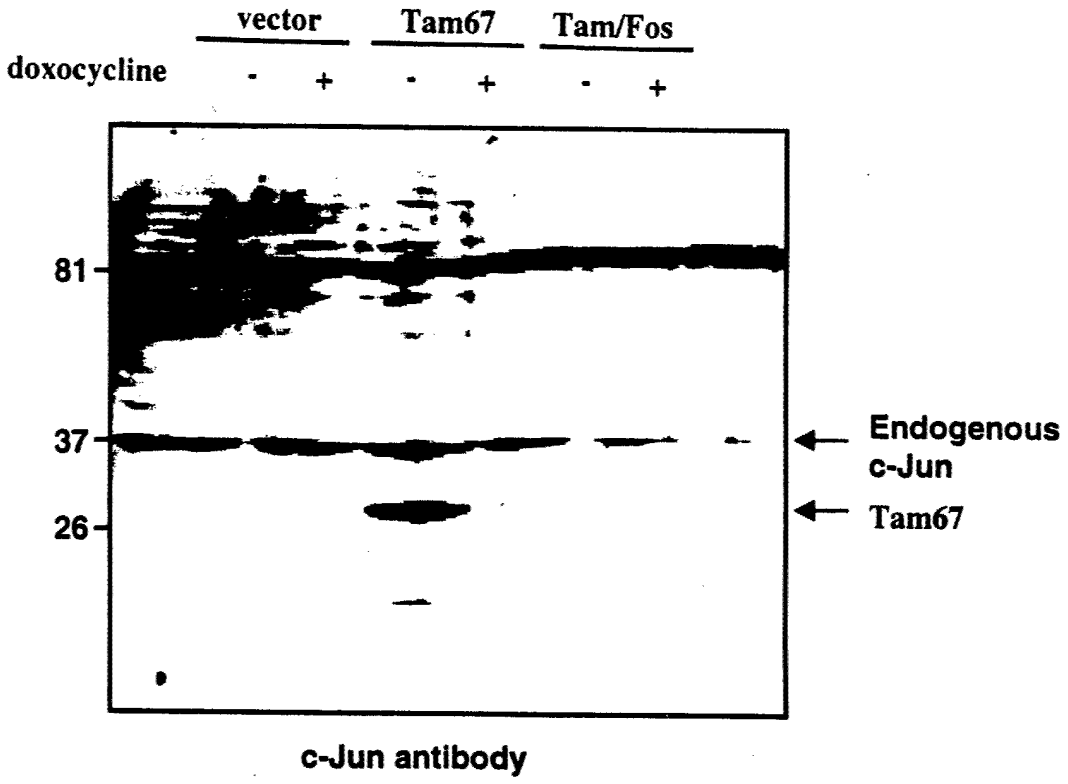

Figure 6: Relative expression of Tam67 and Tam67/Fos in comparison to endogenous c-Jun in MCF-7 cells. MCF-7 cells stably transfected with Tet-regulated expression vectors for vector alone (vector), Flag-tagged Tam67 or Flag-tagged Tam67/Fos were cultured in the absence ('-') or presence ('+') of doxycycline for 6 days. Cell extracts were prepared, resolved by SDS-PAGE, transferred to nitrocellulose and assessed by Western blot for the expression of dominant-negative c-Jun in comparison to endogenous c-Jun with an antibody to c-Jun. This figure shows induction of Tam67 to levels greater than endogenous c-Jun.

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SKF-82958 Is a Subtype-selective Estrogen Receptor-α (ERα) Agonist That Induces Functional Interactions between ERα and AP-1*

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The transcriptional activity of estrogen receptors (ERs) can be regulated by ligands as well as agents such as dopamine, which stimulate intracellular signaling pathways able to communicate with these receptors. We examined the ability of SKF-82958 (SKF), a previously characterized full dopamine D1 receptor agonist, to stimulate the transcriptional activity of ERα and ERβ. Treatment of HeLa cells with SKF-82958 stimulated robust ERα-dependent transcription from an estrogen-response element-E1b-CAT reporter in the absence of estrogen, and this was accompanied by increased receptor phosphorylation. However, induction of ERβ-directed gene expression under the same conditions was negligible. In our cell model, SKF treatment did not elevate cAMP levels nor enhance transcription from a cAMP-response element-linked reporter. Control studies revealed that SKF-82958, but not dopamine, competes with 17β-estradiol for binding to ERα or ERβ with comparable relative binding affinities. Therefore, SKF-82958 is an ERα-selective agonist. Transcriptional activation of ERα by SKF was more potent than expected from its relative binding activity, and further examination revealed that this synthetic compound induced expression of an *AP-1* target gene in a tetradecanoylphorbol-13-acetate-response element (TRE)-dependent manner. A putative TRE site upstream of the estrogen-response element and the amino-terminal domain of the receptor contributed to, but were not required for, SKF-induced expression of an ERα-dependent reporter gene. Overexpression of the AP-1 protein c-Jun, but not c-Fos, strongly enhanced SKF-induced ERα target gene expression but only when the TRE was present. These studies provide information on the ability of a ligand that weakly stimulates ERα to yield strong stimulation of ERα-dependent gene expression through cross-talk with other intracellular signaling pathways producing a robust combinatorial response within the cell.

The effects of estrogens are mediated by the products of two separate genes, one for estrogen receptor-α (ERα)[1] and another for ERβ. Both are members of the nuclear receptor superfamily of ligand-activated transcription factors. The mechanisms by which ERs activate target gene expression in response to estrogen signaling have been the subject of intense investigation since their respective cDNAs were cloned (1, 2). Because of the relatively recent identification of ERβ, the bulk of our knowledge regarding the genomic effects of estrogens is derived from ERα studies. For instance, upon binding to 17β-estradiol (E_2), ERα undergoes a series of biochemical alterations including increased phosphorylation and conformational changes as well as homodimerization and binding of the receptor to its target DNA sequence, the estrogen-response element (ERE; see Refs. 3–5). ERβ also undergoes conformational changes in response to ligand binding (6, 7) and is phosphorylated *in vivo* (8). With respect to DNA binding, ERβ binds to the same consensus ERE that ERα does, although the latter receptor has an ~4-fold higher affinity for this DNA sequence in comparison to ERβ (9, 10).

Whereas many aspects of the regulation of ERα and ERβ transcriptional activity are quite similar (*e.g.* both bind to EREs and activate transcription in response to E_2 binding), a number of differences between these receptors have been noted. For instance, on ERE-containing reporters, ligands such as 4-hydroxytamoxifen exert partial agonist activity on ERα but act as ERβ antagonists (11). This is likely related to differences in the poorly conserved structure and function of the hormone-independent activation function-1 (AF-1) domain that is located in the amino termini of these receptors (11–13). The carboxyl-terminal AF-2 domain is hormone-dependent, reflecting the ability of agonists to bind to the ligand binding domain of the receptor and induce a conformational change that creates a binding site for coactivators such as steroid receptor coactivator-1 (SRC-1) and its related family members (14, 15). Intriguingly, this domain is only ~60% conserved between ERα and ERβ, and small differences in the affinity of these two receptors for ligands such as genistein and 16α-bromo-17β-estradiol have been demonstrated (16, 17). Although several contexts exist whereby the transcriptional activity of ERα is derived predominantly from the AF-1 or AF-2 domains, in most cells the two activation functions work together to bring about a synergistic activation of transcription (18–20). In contrast, the amino terminus of ERβ possesses relatively low transcriptional activity in comparison to ERα, and this region has been shown to repress the activity of the AF-2 domain of ERβ (11–13).

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[1] The abbreviations used are: ERα, estrogen receptor-α; ER, estrogen receptor; AF, activation function; CAT, chloramphenicol acetyltransferase; E_2, 17β-estradiol; ERE, estrogen-response element; IBMX, 3-isobutyl-1-methylxanthine; MAPK, mitogen-activated protein kinase; PR, progesterone receptor; SKF, SKF-82958; SRC, steroid receptor coactivator; TRE, TPA-responsive element; TPA, tetradecanoylphorbol-13-acetate; CRE, cAMP-response element; DMEM, Dulbecco's modified Eagle's medium; sFBS, charcoal-stripped fetal bovine serum; 8-Br-cAMP, 8-bromo-cyclic AMP; CBP, CREB-binding protein.

Estrogen receptors, in addition to their regulation by ligands, can also be activated by extracellular agents that initiate intracellular signal transduction pathways (reviewed in Ref. 21). For instance, epidermal growth factor or insulin-like growth factor-1 treatment of cells results in initiation of a mitogen-activated protein kinase (MAPK) signal transduction cascade leading to phosphorylation of the Ser^{118} phosphorylation site of ERα and stimulation of ERα transcriptional activity (22–24). Similarly, activation of MAPKs by either epidermal growth factor treatment or by transfection of a dominant active form of Ras induces ERβ phosphorylation and transcriptional activity (8, 25), and this is accompanied by a phosphorylation-dependent recruitment of the SRC-1 coactivator (26). In addition to growth factors, insulin, heregulin, 3,3′-diindolylmethane, and the neurotransmitter dopamine can also stimulate ERα transcriptional activity in the apparent absence of ligand (27–31). The latter was among the first agents demonstrated to stimulate ERα transcriptional activity in a ligand-independent manner (31). There is no information on the ability of dopamine to stimulate ERβ transcriptional activity. However, dopaminergic activation is not unique to ERα, because this neurotransmitter also activates the human vitamin D (but not glucocorticoid) and chicken progesterone receptors (31, 32). Furthermore, *in vivo* studies have demonstrated that dopamine receptor agonists administered to the third ventricle of the brain lead to initiation of lordosis behavior, a progesterone receptor (PR)-dependent biological response in rodents (33–35).

Dopamine receptors are members of the G protein-coupled receptor superfamily, and five genes encoding the D1–D5 subtypes of dopamine receptor have been identified (36). Studies with subtype-specific synthetic dopamine receptor agonists indicate that it is the D1 and/or D5 dopamine receptors that stimulate steroid receptor transcriptional activity (33, 34, 37), and this is associated with D1 and D5 dopamine receptor stimulation of intracellular cAMP production (36). The mechanisms by which the dopaminergic cell signaling pathway communicates with ERα are not well defined, but it is presumed that increased ERα phosphorylation contributes to this process. In this regard, it is interesting to note that cAMP signaling pathways stimulate ERα transcriptional activity and phosphorylation (38, 39). The chicken PR is also ligand-independently activated by treatment of cells with dopamine or agents that increase intracellular cAMP levels (31, 40). However, cAMP activation of PR-dependent transcription is not accompanied by increased receptor phosphorylation but rather by an increase in the phosphorylation of the SRC-1 coactivator with which the receptor interacts to stimulate gene expression (41, 42). Taken together, the data support a model in which dopamine and cAMP signaling pathways stimulate gene expression in a receptor-specific manner.

Alterations in the biology of dopamine and its receptors play an important role in a number of human diseases, such as Parkinson's disease, as well as contribute to the reward seeking behaviors associated with cocaine abuse (43–45). The molecular mechanisms of dopamine and dopamine receptor action have therefore been extensively studied, and these efforts have been aided by the identification of high affinity and potent ligands for dopamine receptors. One such compound, SKF-82958 (SKF), is a full dopamine D1 subtype-selective receptor agonist with greater potency than dopamine (46, 47). SKF has also been shown to stimulate the transcriptional activity of ERα in SK-N-SH neuroblastoma and MCF-7 breast cancer cells (27, 37). We therefore used SKF-82958 to determine the ability of dopaminergic signaling pathways to regulate ERβ transcriptional activity. We observed that this D1 receptor-selective agonist stimulated the transcriptional activity of ERα but had negligible agonist activity for ERβ. We also found that SKF-82958 stimulates phosphorylation of ERα to an extent similar to that observed for E_2. However, SKF-82958 competed with E_2 for binding to the receptor, suggesting that it exerts at least some of its effects on ERα transcriptional activity as an ERα agonist. Stimulation of ERα transactivation was greater than that anticipated from its relative binding affinity for ERα, and we therefore examined the ability of SKF-82958 to stimulate intracellular signal transduction pathways. Whereas SKF-82958 did not increase cAMP production, it did stimulate pathways leading to activation of AP-1, a transcription factor known to functionally interact with many steroid receptors (3), and we therefore examined the contribution of AP-1 to SKF-induced ERα transcriptional activity. These studies provide novel information on the ability of a compound to stimulate simultaneously the activity of two transcription factors and in so doing produce robust stimulation of gene expression through a combinatorial response within the cell.

EXPERIMENTAL PROCEDURES

Chemicals—E_2, tetradecanoylphorbol-13-acetate (TPA), and poly-L-lysine were obtained from Sigma. The anti-estrogens, ICI 182,780 and 4-hydroxytamoxifen were gifts from Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK) and D. Salin-Drouin (Laboratoires Besins Iscovesco, Paris, France), respectively. 8-Bromo-cyclic AMP (8-Br-cAMP) and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Research Biochemicals International (Natick, MA) as were dopamine and the synthetic D1 receptor agonist, SKF-82958. All other chemicals were reagent grade.

Plasmids—The mammalian expression vectors for wild type human ERα ($pCMV_5$-hERα) and its corresponding phosphorylation mutants (S104A/S106A/S118A, S118A and S167A) have been described previously (39) as have the plasmids for human ERβ ($pCMV_5$-hERβ (48)), mouse ERα-Y541A (49), c-Jun (pRSV-jun (50)), c-Fos (pBK-28 (51)), and the pRSV-Not control vector (52). Experiments with deletion mutants of ERα used constructs encoding wild type ERα (amino acids 1–595), ERα-N282G (amino acids 1–282), ERα-179C (amino acids 179–595), ERα-3× (amino acids 1–595 with three point mutations, D538A/E542A/D545A), and ERα-179C-3× (amino acids 179–595 with the D538A/E542A/D545A mutations) expressed from the pRST7 vector (20). Plasmids for the SRC-1e, TIF2, RAC3, and CBP coactivators in the pCR3.1 expression vector have been described previously (53). The estrogen-responsive reporter genes, *ERE-E1b-CAT* (54) and *ERE-E1b-LUC* (55), have been used in previous studies, and both contain nucleotides −331 to −87 of the vitellogenin A2 promoter linked upstream of the adenovirus E1b TATA box. The *p-169αCG-CAT* and *p-100αCG-CAT* reporter genes contain portions of the chorionic gonadotropin gene, with or without a cAMP-response element (CRE), respectively, upstream of the chloramphenicol acetyltransferase (CAT) reporter gene (56). The *coll73-CAT* reporter and the *coll60-CAT* reporters contain portions of the collagenase gene upstream of CAT differing in the inclusion or exclusion of a TRE, respectively (57). An expression vector for β-galactosidase, pCMVβ, was obtained from CLONTECH (Palo Alto, CA).

The mammalian expression vector for FLAG-hERα was constructed as follows. The yeast expression vector for human ERα, YEPE2 (58), was digested with *Tth*III, blunted, and subsequently digested with *Kpn*I. The resulting fragment was cloned into the *Bam*HI (blunted) and *Kpn*I sites of pSelect-1 (Promega). The ER cDNA was removed from the resulting vector with *Kpn*I and *Sal*I restriction enzyme digestion and subcloned into the mammalian expression vector, pJ3Ω (59), to create $pJ3\text{-}hER^{Val400}$. The amino-terminal FLAG epitope was created by utilizing a PCR approach. Briefly, a 5′ primer (5′-GGGGTCGACCATGGACTACAAGGACGACGATGACAAGATGACCATGACCCTCCAC) encoding a methionine residue linked to the FLAG epitope sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) and the first six amino acids of human ERα and a 3′ primer (5′-GCGCTTGTGTTTCAACATTCTCC) corresponding to nucleotides 1017–1039 were used to amplify an 844-base pair nucleotide fragment of the ERα cDNA using pSVMTwt:ER as template (30). The resulting PCR product was digested with *Sal*I and *Not*I and substituted for the *Sal*I-*Not*I fragment of $pJ3\text{-}hER^{Val400}$ to create $pJ3\text{-}FLAG\text{-}hER\alpha^{Val400}$. To replace Val^{400} with cDNA encoding the wild type amino acid (Gly^{400}), the *Not*I-*Sac*I fragment of pSVMTwt:ER (corresponding to amino acids 65 to 595) was substituted for the corresponding region of $pJ3\text{-}FLAG\text{-}hER\alpha^{Val400}$ to create pJ3-FLAG-hERα.

Reporter genes lacking the putative TRE were generated from the parent *ERE-E1b-CAT* plasmid by deletion or site-directed mutagenesis. In the former case, a 195-bp fragment of *ERE-E1b-CAT* was removed by digestion with *Nde*I and *Eco0109*I. The resulting vector was blunt-ended with Klenow and religated to yield *ERE-E1b-CAT*(Δ*Nde-Eco*). To remove the putative TRE sequence by site-directed mutagenesis, the *Ssp*I-*Hin*dIII fragment of *ERE-E1b-CAT* was subcloned into pALTER-1 (Promega). By using the PCR Site-directed Mutagenesis System (Invitrogen) and a mutagenic primer, the putative TRE sequence, TGACACA, was mutated to GGACTCA following the manufacturer's recommendations. The latter sequence had been demonstrated previously to prevent AP-1 binding (60). Following sequencing to verify appropriate nucleotide substitutions, a *Nde*I-*Eco0109*I fragment was removed from pALTER-1 and substituted for the comparable region of *ERE-E1b-CAT* to generate *ERE-E1b-CAT(mTRE)*.

Cell Culture, DNA Transfections, and Transactivation Assays—HeLa cells were routinely maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum. DNA transfections were performed by either Lipofectin (Invitrogen) or adenovirus-mediated approaches (61). For transactivation assays, 24 h prior to transfection, 3×10^5 HeLa cells were seeded per well of a 6-well multiple dish in phenol red-free DMEM containing 5% dextran-coated, charcoal-stripped serum (sFBS). For Lipofectin transfections, cells were incubated with the indicated DNAs and Lipofectin according to the manufacturer's guidelines. Six hours later, the DNA/Lipofectin mixture was removed, and cells were fed with phenol red-free media containing 5% sFBS and the indicated treatments, and 24 h thereafter the cells were harvested.

To prepare reagents for adenovirus-mediated transfections, replication-deficient adenovirus dl312 was propagated and covalently modified with poly-L-lysine by the method of Cristiano *et al.* (62) modified as described previously (61). CsCl-purified fractions of the modified virus were stored at −80 C until use. Adenovirus-DNA complexes were prepared by adding the lysine-modified adenovirus to plasmid DNA and subsequently incubating with a 200-fold molar excess of poly-L-lysine (M_r 18,000–20,000). The adenovirus-DNA-lysine complex was then added to the cells at a virus to cell multiplicity of infection of 500:1. After incubation for 2 h, the medium was replaced with phenol red-free DMEM supplemented with 5% sFBS. Hormones and/or other treatments, as indicated, were added to the cells 4 h later, and the cells were then harvested 24 h thereafter.

Assays of reporter gene expression were performed on cell extracts prepared by lysing cells by rapid freeze-thaw or addition of lysis buffer (Promega). CAT activity was measured by a phase-extraction method utilizing [^{3}H]chloramphenicol (Perkin-Elmer Life Sciences) and butyryl-coenzyme A (Amersham Biosciences) as substrates (30, 63). Luciferase activity was measured using the Luciferase Assay System (Promega). Duplicate samples were measured in each experiment, and data are presented as the average ± S.E. of at least three experiments normalized to protein content measured by Bio-Rad protein assay reagent or β-galactosidase.

Relative Binding Affinity Assays—Relative receptor binding affinities were determined *in vivo* as described previously (64). Briefly, the adenovirus-mediated DNA transfer procedure was used to transfect HeLa cells with 0.25 μg/well of the appropriate expression vector (pCMV$_5$-hERα or pCMV$_5$-hERβ). Twenty four hours later, media were aspirated from wells and replaced with phenol red-free DMEM containing 5% sFBS, ~1 pmol of [^{3}H]estradiol (Perkin-Elmer Life Sciences), and increasing concentrations (ranging from 10^{-10} to 10^{-3} M) of either unlabeled E_2, SKF-82958, or dopamine. Following 2 h of incubation at 37 °C, media were aspirated from plates, and cells were washed 3 times in cold PBS and then incubated in 100% ethanol for 15 min at room temperature to extract bound steroid. The amount of ER-bound [^{3}H]estradiol in the ethanol extract was quantified with a Beckman LS 6500 scintillation counter and Biodegradable Counting Scintillant (Amersham Biosciences).

Western Blot Analyses—To assess ER expression, cells were transfected as described above and harvested for Western blot analysis 24 h later. Cell pellets were resuspended in 50 mM Tris buffer (pH 8.0) containing 400 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.2% Sarkosyl, 100 μM sodium vanadate, 10 mM sodium molybdate, and 20 mM NaF, incubated on ice for 60 min, and centrifuged at 12,000 × *g* for 10 min at 4 °C. The resulting supernatant was mixed with SDS-PAGE loading buffer, resolved by 7.5% SDS-PAGE, and electrotransferred to nitrocellulose. Filters were incubated sequentially with primary antibodies against ERα (H222) or the FLAG epitope (M2; Sigma) and the appropriate horseradish peroxidase-conjugated antibody. Immunodetection was performed with enhanced chemiluminescence (ECL) reagents as recommended by the manufacturer (Amersham Biosciences).

^{32}P Labeling and ERα Immunoprecipitation—Cells were transfected with either pJ3-FLAG-hERα or pJ3Ω by the adenovirus method. Eight hours later, media were removed, and cells were rinsed with phosphate-free DMEM and re-fed with phosphate-free DMEM containing 5% dialyzed charcoal-stripped fetal calf serum (HyClone, Logan, UT). Radiolabeled inorganic phosphate (83 μCi/ml media) was added, and cells were incubated for 16 h. Vehicle (ethanol), 1 nM E_2, or 25 μM SKF-82958 was added 90 min prior to harvesting cells. Cells were lysed in 50 mM Tris (pH 8.0) containing 5 mM EDTA, 1% Triton X-100, 0.2% Sarkosyl, 400 mM NaCl, 200 μM sodium vanadate, 10 mM sodium molybdate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml aprotinin, 3 μg/ml leupeptin, 3 μg/ml pepstatin, 20 mM disodium *p*-nitrophenyl phosphate, 25 mM β-glycerophosphate, 5 mM L-Phe-Ala, and 0.15 mM 1,10-phenanthroline for 60 min on ice. Lysates were precleared with rabbit anti-rat IgG and protein A-Sepharose prior to the sequential addition of 5 μg of H222 antibody, 10 μg of rabbit anti-rat IgG, and protein A-Sepharose. The immunoreactive Sepharose complex was washed with 100 mM Tris buffer (pH 9.0) containing 150 mM NaCl, 1% Triton, 1% Tween 20, 20 mM sodium fluoride, 1 mM sodium vanadate, and 10 mM sodium molybdate and eluted with 1 M acetic acid. Samples were resolved by 7.5% SDS-PAGE and electroblotted to nitrocellulose and subjected to autoradiography at −80 °C. Protein levels were subsequently assessed by subjecting this same membrane to Western blot analysis using the anti-FLAG M2 antibody, followed by a secondary antibody of horseradish peroxidase-conjugated sheep anti-mouse IgG. Signals were revealed with ECL methods following the manufacturer's instructions (Amersham Biosciences). The ^{32}P signals were quantitated by a Betagen Betascope 603 Blot Analyzer and normalized to immunoprecipitated protein assessed by Western blot analysis and quantitated by scanning laser densitometry (model 620, Bio-Rad Laboratories).

RESULTS

SKF-82958 Activation of ERα-dependent Gene Transcription—As reported previously (37), the dopamine D1-selective agonist SKF-82958 (±-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrehydro-1*H*-3-benzazepine; see Fig. 1*A*), like dopamine, can stimulate ERα transcriptional activity, and this is inhibited by the pure ER antagonist ICI 182,780 (Fig. 1*B*). Dose-response studies indicated that half-maximal induction of ER-directed gene expression by SKF-82958 occurred at 2 μM (data not shown). In contrast, maximal dopamine induction of ER-directed gene expression occurs at 100–250 μM (23, 30, 31), suggesting that SKF-82958 is a more potent activator of this response. However, the potency (K_m) and maximum efficacy of SKF-82958 induction of cAMP are similar to that for dopamine in rat brain striatum after treatment *in vivo* (46). This discrepancy suggested that there may be mechanistic differences in the ability of SKF-82958 and dopamine to stimulate ERα transcriptional activity.

To investigate this further, relative to the mechanisms of SKF-82958 activation of ERα-dependent gene expression, SKF stimulation of cAMP production in HeLa cells was examined by radioimmunoassay and compared with the ability of SKF to activate ER-dependent gene expression. No correlation was found, as micromolar doses of SKF-82958 failed to elevate significantly cAMP levels (data not shown). To mimic more closely the conditions under which our transactivation assays are performed, the ability of SKF-82958 to stimulate CRE-dependent transcription was assessed. The *−169αCG-CAT* gene is composed of a fragment of the human chorionic gonadotropin gene promoter containing a CRE element, linked upstream of the CAT reporter gene, and is activated by cAMP stimulation of the cAMP-response element-binding protein transcription factor (56). The *−100αCG-CAT* reporter gene that lacks the CRE was used as a negative control. As shown in Fig. 1*C*, CRE-dependent transcription was stimulated by 8-Br-cAMP and, more modestly, by dopamine. However, there was no stimulation of CRE-dependent transcription by E_2 or SKF-82958. These results suggest that SKF-82958 is not acting through stimulation

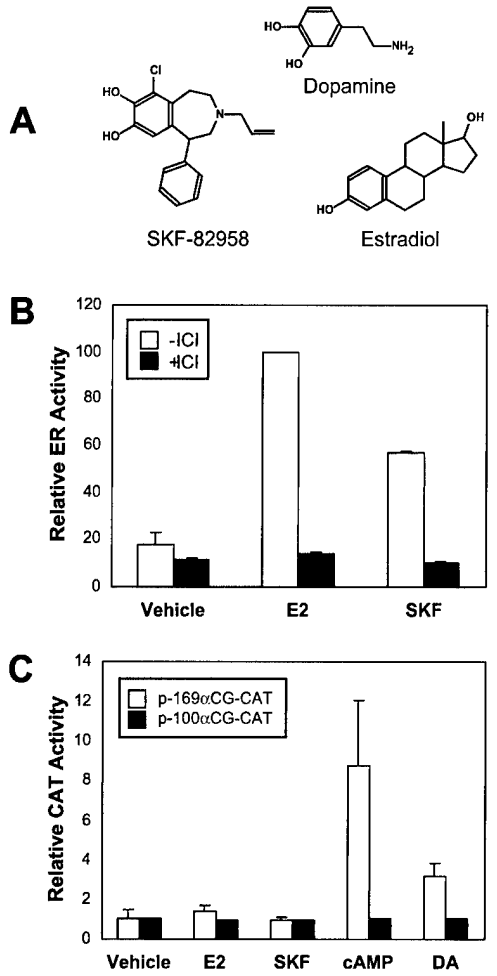

FIG. 1. **SKF-82958 activates ERα-dependent gene expression.** *A*, chemical structures of the compounds used to regulate ERα activity in this study. *B*, activation of *ERE-E1b-Luc* target gene expression by SKF-82958 is ER-dependent. HeLa cells were transfected with expression vectors for ERα ($pCMV_5$-hERβ) and β-galactosidase (pCMVβ), and the *ERE-E1b-Luc* reporter gene and subsequently treated with ethanol (*vehicle*), 1 nM E_2, or 10 μM SKF in the absence or presence of 100 nM ICI 182,780. Data represent the average of three independent experiments ± S.E. *C*, SKF-82958 does not stimulate CRE-dependent transcriptional activity. HeLa cells were transfected with either a CRE-containing (*p-169αCG-CAT*) or CRE-minus (*p-100αCG-CAT*) reporter gene and subsequently treated with ethanol (*Vehicle*), 1 nM E_2, 25 μM SKF, 1 mM 8-Br-cAMP, and 100 μM IBMX (*cAMP*), or 200 μM dopamine (*DA*). Activation data represent the average ± S.E. of three independent experiments.

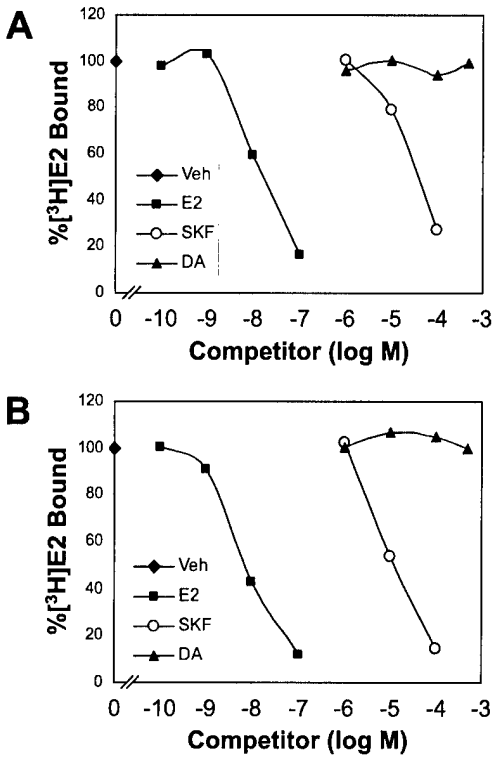

FIG. 2. **SKF-82958 binds to ERα and ERβ.** *In vivo* hormone binding assays of ERα (*A*) or ERβ (*B*) were performed to assess the relative binding affinity of E_2, SKF, or dopamine (*DA*) with respect to competition for [^{3}H]estradiol binding to receptor. Total [^{3}H]estradiol binding in the absence of competitor (♦) is shown for cells treated with ethanol (*Veh*). Values represent the average of duplicate samples from a representative experiment. Similar results were obtained in n = 3–4 independent experiments.

of cAMP-dependent dopaminergic signaling in this system. This result led to a consideration of whether this compound activated ER-dependent gene expression through direct binding to ERα. This question is further underscored by the ring structure of this synthetic D1 receptor agonist (Fig. 1*A*), which is reminiscent of the structures of some ER agonists and antagonists (65).

SKF-82958 Binds to ERα and ERβ but Preferentially Activates ERα—To determine whether SKF-82958 could bind to ERs, whole cell competitive hormone binding assays were performed in HeLa cells transfected with expression vectors for either ERα or ERβ. Cells were incubated with [^{3}H]estradiol and increasing amounts of unlabeled E_2, SKF-82958, or dopamine. The displacement curves for ERα and ERβ indicate that SKF-82958 can compete weakly with estradiol for binding to both forms of ER but that dopamine is unable to do so (Fig. 2, *A* and *B*). The average relative binding affinities of SKF-82958 in comparison to E_2 (100) for ERα (0.077 ± 0.018; n = 4) and ERβ (0.069 ± 0.009; n = 3) are similar and are comparable with those measured by other investigators for low affinity ER agonists such as bisphenol A (16). This result suggests that activation of ER-dependent gene expression may arise through SKF-82958 binding to ERs and serving as a weak receptor agonist, and we therefore wanted to determine whether SKF-82958 could activate both subtypes of ER.

HeLa cells were transfected with expression vectors for human ERα or ERβ and the *ERE-E1b-Luc* reporter gene, which consists of the ERE from the vitellogenin A2 promoter linked to the TATA box sequence of the adenovirus *E1b* gene and luciferase reporter gene. SKF-82958 was not able to activate significantly ERβ-dependent gene expression in comparison to the ability of this compound to stimulate ERα transcriptional activity as shown in Fig. 3*A* or in dose-response studies (data not shown). SKF therefore appears to be an ERα-preferential agonist. To ensure that SKF-82958 induction of ERα-dependent gene expression was not due to ligand stabilization of ERα expression, Western blot analysis of ERα expression in cells treated with vehicle, E_2, and SKF-82958 was performed, and like E_2 and dopamine (30, 53), SKF was found to down-regulate the expression of ERα in HeLa cells (Fig. 3*B*). The ability of SRC family and CBP coactivators to enhance SKF-induced ERα transactivation was also examined. Each was able to significantly enhance the transcriptional activity of ERα (Fig. 3*C*), suggesting that SKF-82958 binding to the receptor allows coactivator-ERα functional interactions.

Characterization of FLAG-tagged ERα—Activation of human ERα by E_2 is accompanied by increased receptor phosphorylation (39, 66). To determine whether SKF-82958 alters the biochemical properties of ERα, the phosphorylation status of the receptor was assessed in HeLa cells treated with SKF-82958 *versus* E_2. First, an expression vector was constructed for

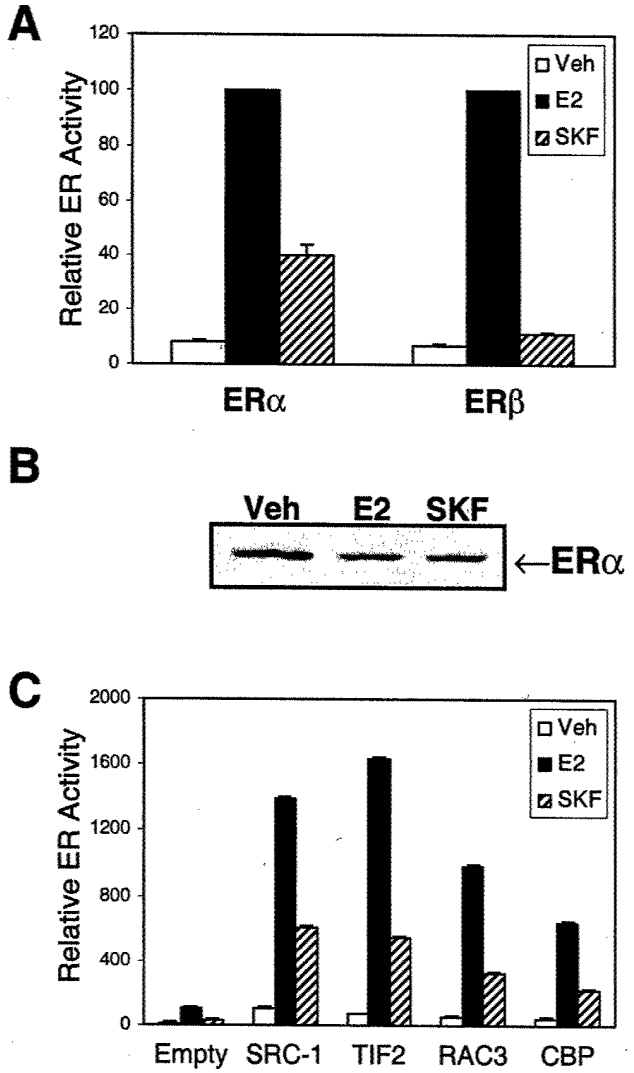

FIG. 3. **SKF-82958 is an ERα-selective activator of transcription.** *A*, HeLa cells were transfected with expression vectors for ERα ($pCMV_5$-hERα) or ERβ ($pCMV_5$-hERβ) along with ERE-E1b-Luc and pCMVβ, and subsequently treated with ethanol (*Veh*), 1 nM E_2, or 10 μM SKF. Data represent the average ± S.E. of four independent experiments. *B*, down-regulation of ERα expression by SKF. Western blot analysis of cell extracts prepared from HeLa cells transfected with an ERα expression vector and subsequently treated with ethanol (*Veh*), 1 nM E_2, or 25 μM SKF. Signals were detected with H222 antibody. *C*, HeLa cells were transfected with *ERE-Elb-Luc* and expression vectors for ERα and β-galactosidase along with plasmids for SRC-1e, TIF2, RAC3, CBP, or the corresponding parental (*empty*) vector, pCR3.1. Cells were subsequently treated with ethanol (*Veh*), 1 nM E_2 or 10 μM SKF. Values represent results from an experiment performed in duplicate and repeated at least three times.

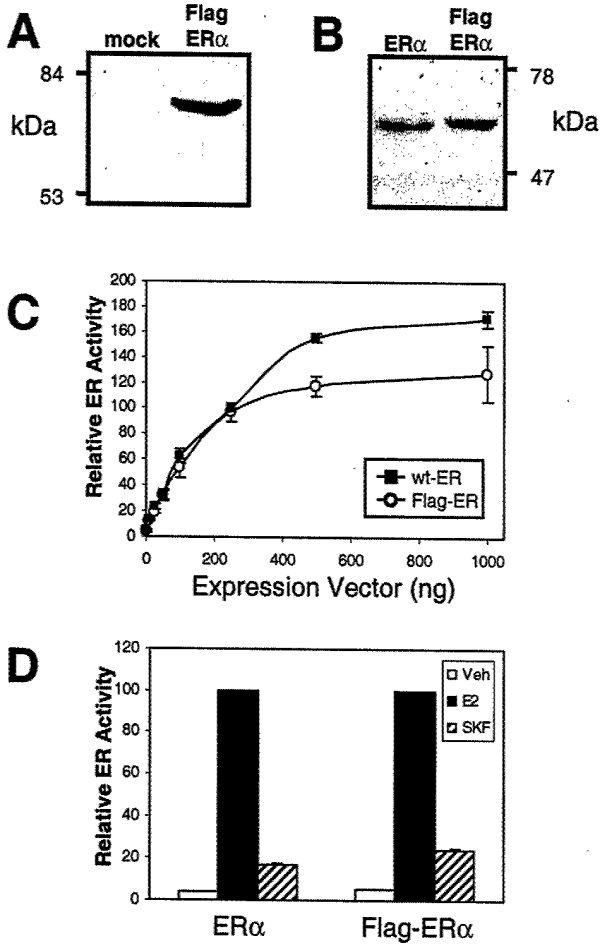

FIG. 4. **Comparison of wild type and FLAG-tagged ERα.** *A*, Western blot analysis of extracts prepared from cells transfected with pJ3-FLAG-ERα or pJ3Ω (*mock*). Blot was probed with anti-FLAG (M2) antibody. *B*, Western blot of wild type and FLAG-ERα expressed in HeLa cells. Blot was probed with anti-hERα (H222) antibody. *C*, dose-response curves for wild type (*wt*; ■) and FLAG-tagged (○) ERα in HeLa cells. Cells were transfected with increasing amounts of expression vectors for wild type or FLAG-tagged ERα, along with *ERE-E1b-Luc* and CMVβgal, and subsequently treated with 1 nM E_2. Data are standardized to the activity of cell lysates prepared from cells transfected with 250 ng of wild type ERα, and represent the mean ± S.E. of three independent experiments. *D*, HeLa cells were transfected with 250 ng of the expression vector for each of the indicated receptor forms along with *ERE-E1b-Luc* and CMVβgal. Cells were treated with ethanol (*Veh*), 1 nM E_2, or 10 μM SKF. Results are standardized to E_2 values and represent the average ± S.E. of three independent experiments.

FLAG-ERα so that distinct antibodies could be used for immunoprecipitation (anti-ERα) and for receptor quantitation by Western blot analysis (anti-FLAG). To demonstrate that the M2 antibody against the FLAG epitope reacted with only FLAG-ERα, cell lysates were prepared from HeLa cells transfected with either pJ3-FLAG-hERα or empty parent vector (pJ3Ω) and subjected to Western blot analysis. The M2 antibody detected an appropriately sized band in HeLa cells transfected with pJ3-FLAG-hERα but not in mock-transfected cells (Fig. 4*A*). In a separate experiment, the hERα antibody, H222, was used to ensure that the protein encoded by the pJ3-FLAG-ERα expression vector was immunoreactive with ERα antibodies. As expected, Western blot analysis demonstrated that the FLAG-ERα migrated with a slightly lower mobility than wild type ERα (Fig. 4*B*).

The transcriptional activity of FLAG-ERα was compared with wild type ERα in transient transfection experiments to ensure that the fusion of the FLAG epitope to the amino terminus of ERα did not adversely affect the relative ability of the chimeric receptor to activate expression of a synthetic target gene. HeLa cells were transfected with the *ERE-E1b-Luc* reporter gene, as well as an expression vector for β-galactosidase (pCMVβ), and increasing amounts (0–1000 ng) of expression vectors for wild type or FLAG-tagged ERα. In cells treated with 1 nM E_2 both receptors exhibited comparable transcriptional activities in the linear portion of the dose-response curve (Fig. 4*C*). Only when very high levels (≥500 ng) of the expression vectors were introduced into cells was a modest reduction in activity observed for FLAG-ERα relative to wild type ERα. Equivalent amounts of vectors for wild type and epitope-tagged ERα were then transfected into HeLa cells, and the ability of

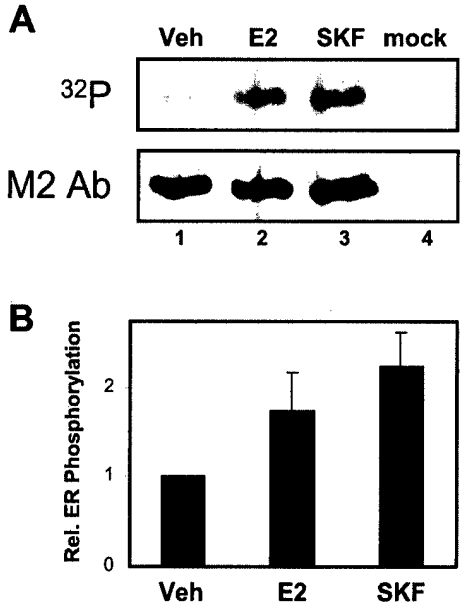

FIG. 5. **SKF-82958 induces ERα phosphorylation.** *A*, HeLa cells transfected with expression vector for FLAG-ERα (*lanes 1–3*) or empty vector (pJ3Ω; *lane 4*) were radiolabeled with [^{32}P]orthophosphate and treated with ethanol (*Veh*), 1 nM E_2, or 25 μM SKF. Receptors were immunoprecipitated with H222 antibody, resolved by SDS-PAGE, transferred to nitrocellulose, and exposed for autoradiography (*top*) and subsequently subjected to Western blot analysis with an anti-FLAG (M2) antibody (*Ab*) (*bottom*). *B*, values represent the average ± S.E. of relative ERα phosphorylation determined in four independent experiments.

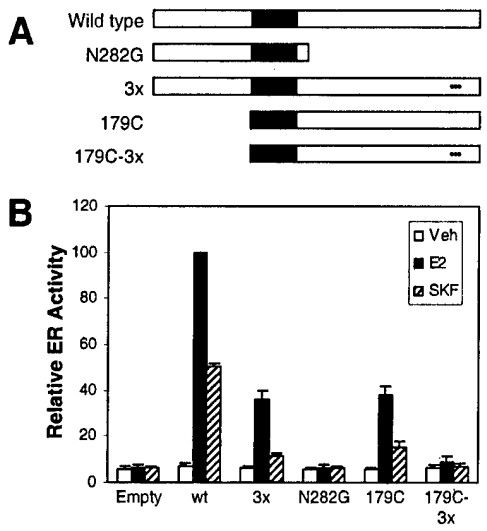

FIG. 6. **The AF1 and AF2 domains of ERα are required for optimal activation of transcription by SKF-82958.** *A*, schematic of ER mutants used in experiments shown in *B*. The location of the D538A/E542A/D545A amino acid mutations are indicated by ●. *B*, HeLa cells were transfected with pRST7 (*empty* plasmid) or pRST7 expression vectors for wild type ERα (*wt*), ERα-3× (*3×*), ERα-N282G (*N282G*), ERα-179C (*179C*), or ERα-179C-3× (*179C-3×*) along with *ERE-E1b-Luc* and pCMVβ. Data are presented as the average ± S.E. of three experiments. Cells were treated with ethanol (*Veh*), 1 nM E_2, or 10 μM SKF. The activity of wild type ERα in the presence of 1 nM E_2 was defined as 100.

each receptor to activate transcription following SKF treatment was determined. Both receptors were activated to an equivalent extent by SKF-82958 (Fig. 4*D*). Taken together these data indicate that the transcriptional activity of FLAG-ERα stimulated by either the natural ligand (E_2) or the weakly estrogenic SKF-82958 is comparable with untagged ERα, and FLAG-ERα was used therefore for analysis in subsequent phosphorylation studies.

SKF-82958 Induces Phosphorylation of FLAG-ERα—To determine whether activation of ERα-dependent gene expression by SKF-82958 is accompanied by alterations in the biochemical properties of the receptor, FLAG-ERα was expressed in HeLa cells using the adenovirus transfection method. Cells were subsequently radiolabeled with [^{32}P]orthophosphate and treated with vehicle, 1 nM E_2, or 25 μM SKF-82958 for 90 min. FLAG-ERα was immunopurified with the H222 anti-ERα antibody, resolved by 7.5% SDS-PAGE, and electrotransferred to nitrocellulose. The resulting blot was subjected to autoradiography to visualize the relative amount of phosphate incorporated into receptor and was subsequently subjected to Western blot analysis with an anti-FLAG antibody (M2) to quantitate relative receptor expression levels. A representative blot indicates that SKF-82958 significantly increased the overall phosphorylation level of ERα relative to ^{32}P incorporation observed in cells treated with vehicle alone (Fig. 5*A*). As expected, the phosphorylation level of FLAG-ERα isolated from cells treated with E_2 was also significantly increased in comparison to basal levels. When protein levels were taken into account, the data averaged from four experiments indicate that E_2 increased ER phosphorylation by 1.7 ± 0.4-fold, whereas SKF treatment increased ER phosphorylation by 2.2 ± 0.4-fold (Fig. 5*B*).

To determine whether any of the known ER serine phosphorylation sites are critical for activation of the receptor by the SKF signal transduction pathway(s), the ability of this putative ligand to stimulate the activity of ER phosphorylation site mutants was assessed (39, 66, 67). Although SKF-82958 was able to stimulate the transcriptional activity of each amino-terminal phosphorylation mutant, activation of S118A (2.7 ± 0.3-fold) and S104A/S106A/S118A ERα (2.5 ± 0.5-fold) mutants was decreased relative to the ability of this compound to activate either wild type (4.5 ± 0.3-fold) or the S167A (4.7 ± 0.2-fold) mutant. These data are consistent with the effects of these mutations on E_2-dependent activity (see Refs. 39 and 66 and our data) and suggest that serines 118 and possibly 104/106 may contribute to, but are not required for, activation of ERα in response to SKF-82958 treatment.

Functional Domains of ERα Required for SKF-82958 Activation—To test more generally the regions of ER required for SKF activation, the ability of this compound to stimulate the transcriptional activity of a series of ER mutants (Fig. 6*A*) was tested. Mutation of the AF-2 domain (D538A/E542A/D545A) in the ERα-3× mutant reduced the ability of E_2 and SKF to stimulate ER activity by ~64 and ~78%, respectively, suggesting that the carboxyl-terminal AF-2 domain contributes to both mechanisms of activation (Fig. 6*B*). An ER mutant lacking the ligand binding and F domains (N282G) was not activated by SKF-82958 or E_2 treatment, and this is in agreement with previous studies in SK-N-SH neuroblastoma cells in which the carboxyl terminus of ERα was required for SKF-82958 activation of target gene expression (37). Deletion of the amino-terminal AF-1 domain reduced E_2-dependent transcriptional activity by ~62% and SKF-dependent gene expression by ~70% in the ERα-179C mutant in comparison to wild type receptor, whereas deletion of the A/B domain in conjunction with the 3× mutation yielded an ER mutant (ERα-179C-3×) unable to activate gene expression in comparison to the empty parent vector. Taken together these data suggest that the amino- and carboxyl-terminal domains of ERα both contribute to receptor activity stimulated by SKF-82958.

SKF-82958 Activates Gene Expression from a TPA-response Element-containing Promoter—A growing body of evidence indicates that most receptors, whether membrane or nuclear, activate and/or interact with numerous signaling pathways. The dual actions of SKF-82958 in activating dopamine D1

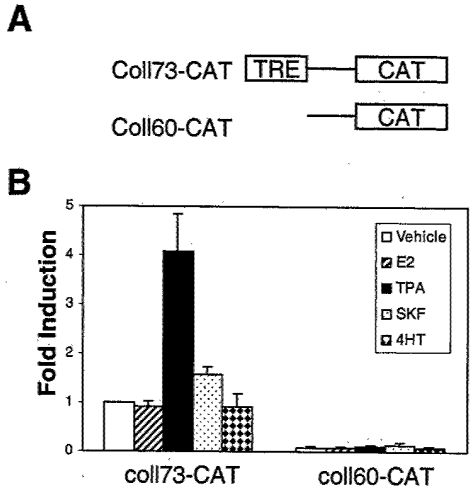

FIG. 7. **SKF-82958 modestly activates TRE-dependent gene expression.** *A*, schematic representation of the *coll73-CAT* and *coll60-CAT* reporter genes used in these experiments. *B*, HeLa cells were plated at a low density (2×10^5 cells/well), switched to media containing 0.5% sFBS, and transfected with *coll73-CAT* or *coll60-CAT* reporter plasmid. Cells were treated with ethanol (*vehicle*), 1 nM E_2, 100 nM TPA, 10 μM SKF, or 100 nM 4-hydroxytamoxifen (*4HT*). Values represent mean ± S.E. for $n = 4–5$ experiments and are expressed as fold induction relative to vehicle-treated cells transfected with *coll73-CAT*.

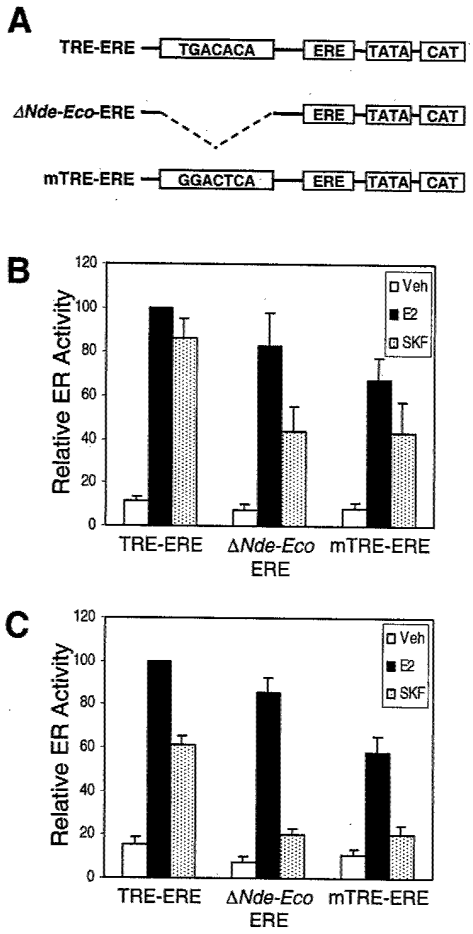

FIG. 8. **An upstream TRE enhances SKF-82958 activation of ERα-dependent gene expression.** *A*, schematic representation of reporter genes used in these experiments. HeLa cells were transfected with expression vectors for wild type ER (pSVMT-wtER) (*B*) or ERα-179C (pRST7-hERα-179C) (*C*) along with *ERE-E1b-CAT* reporter genes encoding a putative AP-1-responsive element (*TRE-ERE*) or lacking this site through deletion (Δ*Nde-Eco*-ERE) or mutation (mTRE-ERE). Cells were treated with the ethanol (*Veh*), 1 nM E_2, or 10 μM SKF. *Bars* represent mean ± S.E. for $n = 4–6$ independent experiments, and values are expressed relative to the CAT activity induced by E_2 treatment from the intact *TRE-ERE-E1b-CAT* reporter in each experiment.

receptors and ERα provided an opportunity to explore the impact of multiple signaling mechanisms induced by a multifunctional activator on nuclear receptor-induced transcription. Although SKF-82958 did not appear to appreciably increase cAMP levels in HeLa cells, activation of dopamine D1 receptors has also been shown to stimulate the activity of protein kinase C (68, 69). We therefore examined whether SKF treatment of cells could stimulate the activity of a sequence-specific transcription factor, AP-1, which is a downstream target of the protein kinase C pathway (70). AP-1 is composed of either homo- or heterodimers within the Jun family (c-Jun, JunB, and JunD) or between members of the Jun and Fos (c-Fos, FosB, Fra1, and Fra2) families (71). HeLa cells were transfected with a *coll73-CAT* reporter, which contains a TRE to which the AP-1 proteins c-Jun and c-Fos bind, or *coll60-CAT* reporter plasmid lacking the TRE (Fig. 7*A*) and treated with ethanol (vehicle), 1 nM E_2, 100 nM TPA, 10 μM SKF-82958, or 100 nM 4-hydroxytamoxifen. TPA strongly induced TRE-dependent gene expression from *coll73-CAT*, whereas neither E_2 nor 4-hydroxytamoxifen resulted in transcriptional activation (Fig. 7*B*). In contrast, treatment with SKF-82958 resulted in weaker, but significant ($p < 0.05$), stimulation of TRE-dependent transcriptional activity. None of the treatments increased transcription from a reporter gene (*coll60-CAT*) lacking the TRE enhancer.

Enhanced SKF-82958 Stimulation of ERα-dependent Gene Transcription by an Upstream TRE—Because SKF weakly stimulated TRE-dependent gene expression and the *ERE-E1b-CAT* reporter gene contains a putative TRE site in the vector backbone ~255 bp upstream of the ERE, we examined the contribution of TRE binding factors to SKF induction of ERα-dependent gene expression. Thus, SKF-82958 or E_2-induced CAT expression were compared in the intact *ERE-E1b-CAT* reporter *versus* constructs in which the putative TRE site was eliminated by deletion (Δ*Nde-Eco*) or point (mTRE) mutagenesis (Fig. 8*A*). The latter point mutant was examined to rule out the possibility that the deletion mutant introduced structural perturbations or removed other cryptic DNA sequences from the reporter that might alter transcriptional responses. As shown in Fig. 8*B*, significant CAT expression was induced by treatment with E_2 or SKF-82958 from either the intact or mutant forms of the *ERE-E1b-CAT* reporter gene. Moreover, the fold induction by E_2 was similar for the three reporter genes, whereas SKF-82958 induction of CAT gene expression was diminished by ~23 and ~28%, when the TRE was deleted or mutated, respectively. The similarity in SKF-82958 effect on gene expression between the reporters generated by deletion *versus* site-directed mutagenesis is consistent with the interpretation that it is the upstream TRE element, rather than some other element or structural alteration, that contributes to the magnitude of SKF-82958-induced ERα transactivation under these conditions. Moreover, in experiments in which a *Cla*I to *Bgl*I linear fragment of the *ERE-E1b-Luc* plasmid encompassing just the ERE, E1b, and luciferase sequences was transfected into HeLa cells with an ERα expression plasmid, SKF stimulation of ERα activity relative to E_2 was 50% the level seen for unaltered (circular) target gene (data not shown). Taken together, these results support the hypothesis that TRE elements in reporter plasmids may enhance, but are not required for, induction of ERα-dependent gene transcription by multifunctional ligands such as SKF-82958.

Because TRE-dependent activity significantly enhanced SKF activation of ERα-dependent gene expression, and because c-Jun has been shown to bind to the amino terminus of ERα (57),

we wanted to ensure that the ligand binding domain and AF-2 could support SKF activation of gene expression in the absence of AF-1. We therefore examined the ability of ERα-179C to be activated by SKF-82958 in the absence of the upstream TRE. As shown in Fig. 8*C*, loss of the TRE site of the reporter and the AF-1 domain of the receptor significantly compromises the ability of SKF-82959 to activate ERα-dependent gene expression, consistent with the interpretation that both the AF-1 and TRE contribute to SKF-induced transcriptional activity. Taken together, these data suggest that SKF-82958 on its own is a weak ERα agonist and that the robust activation seen with full-length receptor is a result of the synergistic activation of ERα and cellular factors, such as c-Jun or c-Fos, that can bind to the TRE (60).

Effect of AP-1 Overexpression on ERα Transactivation by SKF-82958—The above observations suggest that transcription factors able to interact with the TRE-binding site can contribute to ERα-mediated gene expression stimulated by SKF-82958. Protein-protein interactions have been reported between the AP-1 protein c-Jun and ER, but not between c-Fos and ER, and occur principally through the amino-terminal AF-1 domain of the ER protein (57). To investigate further the ability of SKF to activate synergistically ER/AP-1-dependent transcription, we tested the hypothesis that increased Jun/Fos expression would enhance SKF-82958 activation of ERα-dependent transcription. HeLa cells were cotransfected with expression plasmids for c-Jun, c-Fos, or equivalent levels of c-Jun + c-Fos (12.5–100 ng/well), with total DNA/well maintained constant by altering the levels of cotransfected empty plasmid. Jun overexpression resulted in strong and significant increases in basal, E_2, and SKF-82958-induced transcription from *ERE-E1b-CAT* but not from reporter genes lacking the TRE (*ΔNde-Eco*), suggesting that c-Jun-activated transcription was primarily dependent on the TRE of the intact reporter and not through binding to ERα (Fig. 9*A*). Fos overexpression resulted in only very modest increases in the effects of E_2 and SKF-82958, with no significant effect on basal activity (Fig. 9*B*). The result from the combination of c-Jun with c-Fos was similar to that of c-Jun alone (Fig. 9*C*). In all experiments performed with the *ERE-E1b-CAT*(*ΔNde-Eco*) reporter construct lacking the TRE, no significant increases in transcriptional activation were induced by AP-1 overexpression (Fig. 9, *A–C*), suggesting that the TRE-binding site was required for strong AP-1 effects.

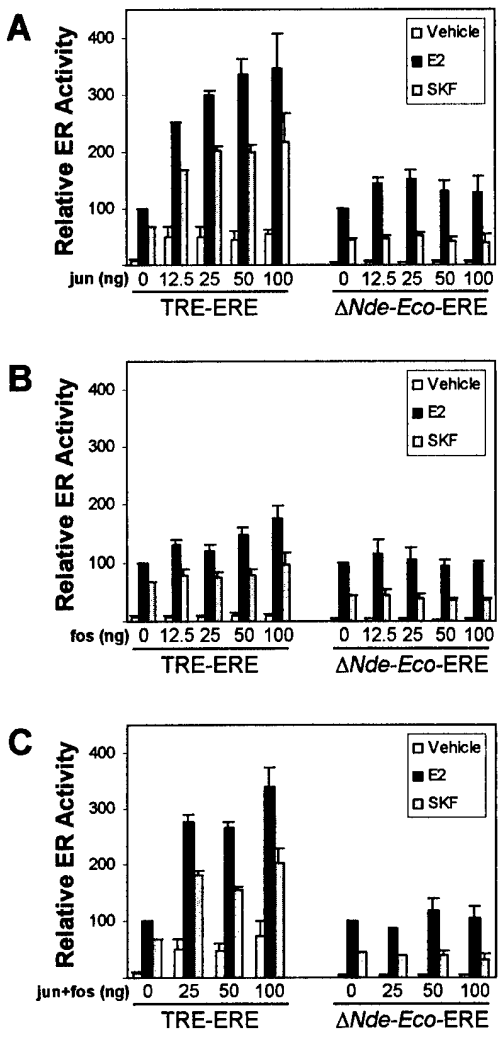

FIG. 9. **Overexpression of c-Jun enhances ER activity stimulated by E_2 or SKF-82958.** HeLa cells were cotransfected with increasing concentrations of expression plasmid for c-Jun (*A*), c-Fos (*B*), or equivalent amounts of c-Jun and c-Fos (*C*) along with *pSVMT-wtER* and *ERE-E1b-CAT* reporter genes with (TRE-ERE) or without (*ΔNde-Eco*-ERE) a TRE. Total DNA levels were normalized in each group by cotransfecting appropriate levels of the empty plasmid pRSV-Not. Transfections were done 6 h prior to addition of the indicated agonists, with harvest following 18 h thereafter. Cells were treated with ethanol (*vehicle*), 1 nM E_2, or 10 μM SKF. *Bars* represent mean ± S.E. for $n = 3$ independent experiments, and values are expressed relative to the CAT activity (100) induced by E_2 treatment from *ERE-E1b-CAT* in each experiment. Analysis of variance indicated that (*a*) c-Jun overexpression, both in the presence and absence of cotransfected c-Fos, significantly elevated basal ($p < 0.001$), and E_2- ($p < 0.01$) and SKF-induced ($p < 0.001$) transcriptional activation from ERE-E1b-CAT, but not from the TRE deletion mutant; (*b*) c-Fos overexpression resulted in modestly significant ($p < 0.05$) increases in E_2- and SKF-induced transcriptional activity from the intact reporter.

DISCUSSION

The relatively high concentration of SKF-82958 required to achieve ERα transcriptional activity in comparison to dopamine D1 receptor activation suggested that this compound was an ERα agonist, and our relative binding affinity analyses demonstrated that SKF competed with E_2 for binding to either ERα or ERβ. However, the results obtained in this study demonstrate that SKF-82958 stimulates the transcriptional activity of ERα, but not ERβ, and therefore SKF-82958 is an ERα-selective agonist. Intriguingly, our studies also demonstrated that SKF stimulates the transcriptional activity of AP-1 and provides evidence that in the appropriate promoter context activation of target gene expression by SKF is the combinatorial result of AP-1 and ERα activation. Understanding the role of AP-1 in SKF-dependent ERα transactivation is particularly important given the ability of SKF to activate both transcription factors. In so doing, the results of these studies provide an example of how other transcription factors can seemingly enhance the potency of weak ER ligands.

Although SKF-82958 is a full agonist of dopamine D1 receptors, it failed to stimulate increases in intracellular cAMP, nor was it able to stimulate CRE-dependent gene expression in our HeLa cells. We had previously shown that dopamine treatment of HeLa cells increased cAMP levels in a dose-dependent manner *in vitro* (30), and the inability of SKF to do so here was unexpected. Although SKF induction of cAMP in SK-N-SH cells had not been characterized, the protein kinase A inhibitor, H89, partially blocked ERα transactivation by SKF-82958 (37), suggesting that a cAMP signaling transduction pathway was playing a role in these cells. Similarly, H89 reduced SKF induction of ER transcription activity in MCF-7 cells (27). These reports are consistent with the ability of SKF to stimulate adenylate cyclase and cAMP production via the dopamine D1 receptor (46, 47), and it is possible that in these cell models ERα transactivation by SKF is at least partially cAMP/protein kinase A-dependent and/or that H89 is inhibiting the activity of

other signaling pathways able to cross-talk with ERα or AP-1. Indeed, whereas H89 effectively inhibits protein kinase A, it also blocks the activity of other kinases including protein kinase B (Akt) and mitogen-and stress-activated protein kinase-1 (72).

The inability of SKF to stimulate ERβ transcriptional activity is unlikely to be due to the minor differences in the relative binding affinities of this compound for ERα and ERβ. A large number of naturally occurring substances, as well as pharmacological and environmental agents, bind to ERs (16, 17). The crystal structures of receptors complexed with E_2, diethylstilbestrol, raloxifene, or 4-hydroxytamoxifen and molecular modeling studies suggest that binding of a phenolic group to the A-ring binding pocket of the ligand binding domains of the receptors is a common feature (14, 73–75). Whereas SKF-82958 does not possess a simple phenolic ring characteristic of many ER ligands (Fig. 1*A*), it does have a hydroxyphenolic ring with a large, bulky chlorine substituent. Based on the ability of C (2) substituted derivatives of E_2 and estrone (2-hydroxyestradiol and 2-hydroxyestrone, respectively) to have severely reduced relative binding affinities for ERs (16, 17) and the chlorine atom on SKF-82958, it was unexpected that SKF would inhibit E_2 occupancy of the ligand binding pocket. This result is perhaps even more surprising in view of the inability of dopamine to bind to ERα or ERβ, because dopamine also possesses a hydroxyphenol ring. However, it is possible that the remainder of the dopamine molecule is of insufficient size to interact with other regions of the ligand binding pocket required for high affinity binding. It will be interesting to determine whether chemical derivatives of SKF-82958 can be generated with increased receptor affinity.

There is significant interest in identifying ER subtype-selective agonists and antagonists, and several investigators have made progress in identifying and characterizing such compounds. These include a *cis*-diethyl-substituted tetrahydrochrysene that has a 4-fold preferential binding affinity for ERβ and is an ERα agonist and complete ERβ antagonist (76), and a methoxychlor metabolite that inhibits estrogen-induced ERβ activity, yet stimulates the transcriptional activity of ERα (77). Potency-selective agonists have also been identified such as pyrazole, which has a 120-fold greater potency for stimulating ERα activity in comparison to ERβ (76), and A-ring reduced metabolites of the 19-nor synthetic progestins, norethisterone and Gestodene, which have at least a 100-fold greater potency for ERα in comparison to ERβ transcriptional activity (78). In addition to these compounds, differences in the ability of steroidal derivatives and non-steroidal phytoestrogens to bind to ERα and ERβ have also been reported (16, 17). Moreover, the differences in the relative agonist and antagonistic activity of several of these novel compounds have been found to correlate with changes in the conformation of the receptors and their ability to bind to SRC family coactivators (79). For instance, the ERα agonist propylpyrazole triol induces an agonistic conformational change in ERα and promotes interaction of this receptor with SRC-1, GRIP1, and ACTR but does not promote interaction of ERβ with these coactivators. We have demonstrated that SRC family coactivators as well as the general coactivator CBP can enhance SKF-induced ERα transactivation, and this is consistent with SKF inducing a conformational change able to promote ERα-coactivator interactions.

By having established that SKF-82958 is an ERα-selective agonist, we examined the mechanism(s) by which it stimulated ERα-dependent gene expression. Deletion of the amino-terminal A/B domain of ERα indicates that the AF-1 domain is not required for SKF-82958 activation of ERα-dependent gene expression nor is a fully functional AF-2 as demonstrated by data from the ERα-3× mutant. However, both these mutations reduce the relative ability of ERα to activate gene expression, and the AF-1 and AF-2 regions are therefore required to yield a full response to SKF stimulation as has been shown in other contexts for E_2 and SKF (20, 37). Deletion of the entire ligand binding domain confirms that SKF-induced ERα transcriptional activity involves the carboxyl terminus of the receptor. As noted above, mutations of the core domain of AF2 reduced, but did not block, the ability of SKF-mediated signaling pathways to activate gene expression, except when combined with deletions of the A/B domain of the receptor. This supports the supposition that SKF activation of ERα transcriptional activity requires the cooperative effects of both the amino- and carboxyl-terminal domains. The inability of SKF to stimulate ERβ transcriptional activity is interesting in view of the contributions of the AF-1 domain of the ERα to this response and differences in the structure and relative transcriptional activity of the AF-1 domains of the two ER subtypes (11–13). It should also be noted that the lack of ERβ transactivation by SKF is not due to an inability of ERβ to interact functionally with AP-1, as we have observed this mechanism in the context of cAMP signaling pathways.[2]

Stimulation of ERα transcriptional activity by SKF is accompanied by increases in the levels of receptor phosphorylation that are similar to those induced in parallel experiments by E_2. However, the enzyme(s) responsible for this post-translational modification and the residue(s) within ERα that are phosphorylated following SKF treatment remain undefined. The similarity of SKF- and E_2-induced phosphorylation of ERα does not correlate with the relative ability of these two compounds to activate the transcriptional activity of this receptor, and this suggests that SKF-induced phosphorylation of ERα may not be important for this process. Although E_2 and growth factor signaling pathways able to stimulate ERα activity induce receptor phosphorylation (4, 21), so do the ERα antagonists, ICI 164,384 and 4-hydroxytamoxifen (39, 66). Taken together, these data suggest that the role of receptor phosphorylation in ligand-induced ERα function may be quite complex and possibly ligand-specific. Alternatively, it is possible that signal transduction pathways initiated by SKF-82958 (see below) could affect receptor-dependent gene expression by phosphorylating coactivators and altering their intrinsic transcriptional activity. For instance, 8-Br-cAMP treatment of COS-1 cells phosphorylates SRC-1 and stimulates its intrinsic transcriptional activity (42). Similarly, growth factor signaling pathways increase the transcriptional activity of the GRIP1 and AIB1 coactivators (80, 81) and cAMP and MAPK signaling pathways increase CBP activity (82, 83). Thus, SKF-induced, ERα-dependent gene expression may also be influenced by SKF-induced alterations in coactivator function.

The ability of SKF to stimulate AP-1 activity contributes to the ability of this compound to stimulate ERα-dependent gene expression on the *ERE-E1b-CAT* reporter gene. Activation of AP-1, however, is insufficient to stimulate CAT activity from this reporter in cells lacking ERα (see Fig. 6*B*). Several lines of evidence indicate that the TRE site contributes to the magnitude of target gene expression by ERα and SKF-82958. First, this synthetic dopamine receptor agonist did activate transcription from a TRE-dependent reporter in the absence of cotransfected ER. Moreover, eliminating a functional AP-1 element ~255 bp upstream from the ERE-E1b-CAT reporter sequence, either by deletion or site-directed mutagenesis, significantly reduced the ability of SKF to stimulate ER transactivation. Interactions between ERα and c-Jun are mediated via

[2] K. M. Coleman and C. L. Smith, unpublished data.

the amino terminus of ERα (57), and eliminating both the upstream AP-1-binding site from *ERE-E1b-CAT* and the AF-1 domain of ERα severely compromised the ability of SKF to activate ERα-dependent gene expression, suggesting that the A/B domain contributes to this activity through its ability to interact with AP-1 and/or accessory transcription factors that link AP-1 and ERα function.

Although steroid receptors can activate the transcription of target genes containing only their response elements and minimal promoters such as TATA boxes, natural target gene promoters are significantly more complex and contain binding sites for many different transcription factors. Regulation of target gene expression is therefore a result of the coordinate regulation of the activity of all transcription factors that can bind to a target promoter, and for this reason, it is important to examine the interaction between AP-1 and ERα. The mechanisms by which SKF enhanced activation of the TRE (*coll73-CAT*) reporter gene are not defined but could be mediated by increased expression of AP-1 transcription factors and/or their activation by signal transduction pathway-induced post-translational modifications (*e.g.* phosphorylation (71, 84)). However, we demonstrated that the magnitude of SKF-dependent ERα transactivation paralleled the relative levels of c-Jun expression (*i.e.* enhanced when c-Jun was overexpressed) confirming that SKF effects dependent on the TRE site are mediated by AP-1. There seems to be a preferential role for c-Jun in this system, because its overexpression resulted in a substantial enhancement of overall transcriptional activity, whereas c-Fos overexpression only modestly enhanced ERα-dependent transactivation. Alternatively, it is possible that other Fos family members may better stimulate ERα activity, analogous to the situation where the ability of E_2 to stimulate or repress AP-1 activity appears to correlate with the relative expression of the Fos family member Fra-1 (85).

These effects of either c-Jun or c-Fos were greatly diminished on *ERE-E1b-CAT* reporters lacking the upstream TRE site. This is important because it suggests that AP-1 interaction with ERα in the absence of TRE DNA-binding site makes very modest contributions to ERα-dependent gene expression. These relationships were particularly well demonstrated when SKF-dependent ERα transactivation of the *ERE-E1b-CAT* TRE site mutants was compared in the presence of wild type ER *versus* the ER mutant lacking the AF-1 domain (Fig. 8). Under these conditions, which limit the contribution of AP-1 both through its DNA-binding site and through protein-protein interactions with ERα, E_2-, and SKF-82958-induced ERα transactivation were substantially diminished. Collectively, these observations are consistent with the hypothesis that AP-1 enhances SKF-dependent ER transactivation both by AP-1/TRE interaction and by protein/protein interaction between the ER and AP-1 proteins. Whether this latter interaction is direct or is indirectly mediated through other proteins such as coactivators is presently unknown.

The interactions between ERs and AP-1 are complex, and using reporters containing only AP-1-binding sites, other investigators have demonstrated two pathways for ER activation of AP-1-dependent gene expression (reviewed in Ref. 3). There appears to be an activation function-dependent pathway that estrogen- or anti-estrogen-liganded ERα utilizes, whereas ERβ stimulates AP-1 activity in an activation function-independent manner (57, 86). The results of our study suggest that AP-1 can stimulate the activity of ERα activated by a weak agonist such as SKF-82958 or as expected with the full agonist, E_2 (57), indicating that these two classes of transcription factors have the ability to regulate each other's transcriptional activity. This also suggests that the ability of any given ER ligand to activate receptor-dependent gene expression may vary depending on the presence of DNA-binding sites for other transcription factors that can functionally interact with the ER and/or that the ligand may regulate. Because ERs have been reported to interact functionally with AP-1 (discussed above), as well as Sp1, NF-Y, and USF (87, 88), many possible regulatory combinations would seem to be possible, leading to complex regulation of ER-dependent gene expression. Taken together, these results suggest that the ability of pharmacological and environmental compounds to exert estrogen-like effects may need to take into account the activities from other transcription factors able to interact functionally with ERα.

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Mechanistic Differences in the Activation of Estrogen Receptor-α (ERα) and ERβ-Dependent Gene Expression by cAMP Signaling Pathway(s)

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SUMMARY

Although increases in intracellular cyclic adenosine monophosphate (cAMP) can stimulate estrogen receptor-α (ERα)-dependent transcription in the absence of exogenous hormone, no studies have addressed whether ERβ could be similarly regulated. This was therefore assessed in transient transfection assays in which forskolin plus 3-isobutyl-1-methylxanthine, which increases cAMP levels, stimulated the transcriptional activities of both ERα and ERβ; this effect was blocked by the protein kinase A inhibitor, H89, and was dependent on an estrogen response element (ERE). A TPA response element (TRE) located 5′ to the ERE was necessary for cAMP-dependent activation of gene expression by ERβ, indicating that this ER subtype requires a functional interaction with TRE-interacting factor(s) to stimulate transcription. In contrast, the TRE site contributed to, but was not required for cAMP activation of full-length ERα indicating a difference in the ability of these two ER subtypes to be activated by this signaling pathway. The ERα EF domains which lack all known c-Jun interaction sites are sufficient for its cAMP-dependent activation provided a TRE site is present in the target gene, indicating that the interaction between ERs and TRE-binding factors such as c-Jun may be indirect. The p160 and CBP coactivators stimulate cAMP-induced ERα and ERβ transcriptional activity, and may mediate this interaction since they are known to bind ERs and AP-1 proteins. Mutation of the two cAMP-inducible SRC-1 phosphorylation sites important for cAMP activation of chicken progesterone receptor (PR) did not specifically impair cAMP activation of ERα. Moreover, while mutation of all seven known SRC-1 phosphorylation sites reduced coactivation of ERα, this again, was not specific for cAMP activation. Taken together, these data indicate that cAMP signaling utilizes distinct mechanisms to stimulate ERα and ERβ transcriptional activity.

INTRODUCTION

The biological effects of estrogens are mediated by two estrogen receptors (ERα and ERβ)[1] that belong to a large superfamily of nuclear hormone receptors. These ligand-regulatable transcription factors possess six structural domains labeled A through F (1). The A/B domain encompasses activation function-1 (AF-1); the C and D domains correspond to the DNA binding domain (DBD) and the hinge region, respectively; the E region encompasses a second activation function (AF-2) and an overlapping ligand binding domain; while the F domain, located at the extreme C-terminus, is thought to play a modulatory role in ER activity. Both estrogen receptors possess similar binding affinities for estradiol and their cognate DNA binding site (estrogen response element; ERE), which is likely due to the high degree of sequence homology that they share in their ligand and DNA binding domains (2-6). Whereas the AF-2 domain of each receptor is regulated by ligand-induced changes in receptor conformation, the activities of the poorly conserved AF-1 domains are ligand-independent and can be modulated by phosphorylation (7-9). Notable for ERα, in most cases the AF-1 and the AF-2 domains interact functionally to enhance transcription in a cooperative manner (7, 10).

In the best-studied mechanism of ERα and ERβ activation, hormone diffuses into the cell, binds to the receptor and induces a conformational change in the receptor's ligand binding domain (1). Receptors, bound to their EREs either as ERα or ERβ homodimers or ERα:ERβ heterodimers, can then recruit coactivators to the promoter region of estrogen target genes via their interaction with the receptor's activation domains (2, 3, 11, 12). There, these molecules can stimulate transcription by bridging ERs to the general transcriptional machinery and promoting the formation of a stable pre-initiation complex (13, 14). Various coactivators also possess

ubiquitin ligase, arginine methyltransferase or histone acetyltransferase enzymatic activities that may facilitate chromatin remodeling and gene activation (15-18).

In addition to this relatively well-characterized mode of activation, ERα can be activated via the cyclic adenosine monophosphate (cAMP) signaling pathway in the apparent absence of estrogens [reviewed in (19)]. In MCF-7 cells, endogenous ERα target genes, including the progesterone receptor [PR; ref. (20)], pS2 (21), Liv-1 (21), and cathepsin-D (22) can be stimulated either with the cAMP analogue, 8-bromo-cAMP (8Br-cAMP), or a combination of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) and cholera toxin, which increases cAMP production via a G-protein-mediated signal transduction pathway. Importantly, in these experiments as well as in later studies the cAMP-induced responses could be inhibited by treatment with the pure antiestrogen, ICI 164,384, signifying their receptor dependence (20-22). Demonstrating the need to transfect ERα into cells lacking endogenous receptor further supported the requirement for receptor (23).

In addition to these ERα studies a number of reports indicate that the transcriptional activities of several other nuclear receptors can be modulated by the cAMP signaling pathway. For example, the chicken PR (24), androgen receptor (25), retinoic acid receptor (26), retinoid X receptor (27), and peroxisome proliferator-activated receptor-δ (28) can be activated by cAMP signaling, demonstrating that this mode of ligand-independent activation is not exclusive to ERα. However, human PR (29) cannot be ligand-independently activated via this mechanism, nor can the unliganded glucocorticoid receptor, although cAMP stimulation increases the hormone-dependent responses of these receptors (30, 31). Collectively, these reports demonstrate the specific nature by which the cAMP signaling pathway can cross-talk with different nuclear receptors.

Some progress has been made in the effort to understand the mechanisms involved in cAMP activation of nuclear receptor-dependent transcription. Whereas an increase in receptor phosphorylation accompanies the cAMP-mediated activation of ERα (32, 33), there is no increase in chicken PR phosphorylation associated with its activation in cells treated with 8Br-cAMP (34). Rather, cAMP/PKA signaling enhances chicken PR-dependent transcription, in part, by increasing phosphorylation of a receptor-interacting coactivator, steroid receptor coactivator-1 (SRC-1), and thereby promotes a more stable interaction between this coactivator and p300/CBP-associated factor (P/CAF) and facilitates functional synergism between SRC-1 and the CREB binding protein, CBP (35). Although it is still unknown how the unliganded ERα is activated by cAMP/PKA, there is evidence that the transcription factor, CREB, can functionally interact with ERα and thereby mediate synergism between the E2-dependent and cAMP-dependent signaling pathways (36).

The identification of ERβ has increased our awareness of the diversity of potential mechanisms by which ER-dependent and estrogenic responses may be achieved (6). Notably, the relative magnitude of ERα- and ERβ-mediated estrogen activation of ERE-containing reporters typically varies depending on the cell type and promoter context (37). In the absence of ligand, both ERα- and ERβ-dependent transcription can be modulated by a mitogen-activated protein kinase (MAPK) signaling pathway (8, 9, 38). It is unknown, however, whether ERβ can be activated by the cAMP/PKA signaling pathway, and we therefore examined this using transient transfection assays and synthetic agents that increase intracellular cAMP levels. In so doing, we have defined mechanistic differences between cAMP activation of ERα and ERβ, particularly with respect to the influence of cross-talk with AP-1 transcription factors. Moreover, we report that all of the p160 coactivators, as well as CBP, can coactivate ERα and

ERβ transcriptional activity stimulated by cAMP pathways and demonstrate that the relative importance of SRC-1 phosphorylation to cAMP activation of gene expression is receptor dependent.

EXPERIMENTAL PROCEDURES

Chemicals

17β-Estradiol (E2), 3-isobutyl-1-methylxanthine (IBMX), and N-{2-[p-bromocinnamylamino]-ethyl}-5-isoquinolinesulfonamide (H89) were obtained from Sigma Chemical Company (St. Louis, MO). Forskolin was obtained from Calbiochem (San Diego, CA).

Plasmid DNAs

The mammalian expression vectors for human ERα, $pCMV_5$-hERα (32) and pCR3.1-hERα (16), and human ERβ, $pCXN_2$-hERβ (39) were described previously. The synthetic target genes pERE-E1b-CAT (40), pE1b-CAT (40), pERE-E1b-CAT(mTRE) (41), 17mer-E1b-CAT (42), and pS2-CAT (43) were used in previous studies, as were pATC0, pATC1, and pATC2 (44). The pCR3.1-hSRC-1a (45) and pCR3.1-hSRC-1a-$Ala^{1179/1185}$ (35) expression plasmids have been published, as were the pCR3.1-TIF2, pCR3.1-RAC3, and pCR3.1-CBP vectors (46). The pBind expression plasmid encoding the GAL4 DBD (amino acids 1-147) was obtained from Promega Corporation (Madison, WI).

The SRC-1a phosphorylation mutant, pCR3.1-hSRC-1a-7Ala which has alanine substitutions at positions 372, 395, 517, 569, 1033, 1179, and 1185 was generated using the Stratagene QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and the appropriate

mutagenic primers. The plasmids were sequenced to ensure that errors did not occur during mutagenesis. The constructs for hERα-179C (pCR3.1-hERα-179C) and hERβ-143C (pCR3.1-hERβ-143C) were made by PCR using the primers 5′-ACCATGGCCAAGGAGACTCGCTACTGT-3′ and 5′-CTCTCAGACTGTGGCAGGGAAACC-3′ to amplify the segment of $pCMV_5$-hERα encoding amino acids 179 to 595 and the primers 5′-ACCATGAAGAGGGATGCTCACTTCTGC-3′ and 5′-GCGTCACTGAGACTGTGGGTTCTG-3′ to PCR amplify the segment of $pCXN_2$-hERβ encoding residues 143 to 530, respectively. Each of the resulting PCR fragments was subcloned into the pCR3.1 expression plasmid using the TA cloning kit (Invitrogen Life Technologies, Carlsbad, CA). The expression plasmids encoding Gal-ERαEF (pBind-ERαEF) and Gal-ERβEF (pBind-ERβEF) were generated by PCR using the primers 5′-GGGATCCGTAAGAAGAACAGCCTGGCCTTGTTCC-3′ and 5′-TCTAGAGACTGTGGCAGGGAAACCCTCTGCC-3′ to amplify the segment of $pCMV_5$-hERα corresponding to amino acids 302 to 595 and the primers 5′-CGGGATCCGAGTGCGGGAGCTGCTGCTGG-3′ and 5′-ATAGTTTAGCGGCCGCTCACTGAGACTGTGGGTTCTG-3′ to amplify the portion of $pCXN_2$-hERβ-encoding amino acids 254 to 530. Each of the resulting fragments was subcloned into the pCR3.1 expression plasmid via the TA cloning kit and subsequently transferred to the pBind expression vector via a *BamH*1-*Xba*1 restriction fragment for pBIND-ERαEF and *BamH*1-*Not*1 fragment for pBIND-ERβEF. The pCR3.1-Flag-hERα construct was generated by PCR using a 5′ primer (5′-GATATTGCTAGCATGGACTACAAGGACGACGATGACAAGACCCTCCACACCAAAGC

ATCT-3′), which incorporated the Flag epitope (underlined), and 5′-CGCCGCAGCCTCAGACCCGGGGCC-3′ to amplify the 5′ region of a hERα cDNA within pCR3.1-hERα, and the resulting PCR fragment was subtituted back into the pCR3.1-hERα expression vector via *Nhe*I and *Xma*I restriction sites. The pCR3.1-3xFlag-hERβ expression vector was constructed as follows: first, the coding region for hERβ was removed from $pCXN_2$-hERβ via a partial digest with *EcoR*I and transferred to pCR3.1, which yielded pCR3.1-hERβ. The 5′ primer (5′-TGACCGTAGCATGGACTACAAAGACCATGACCGTGATTATAAAGATCATGACATC GATTACAAGGATGACGATGACAAGGATATAAAAAACTCACCATCT-3′), which encompasses a coding sequence for three Flag peptides in tandem (underlined), and 3′ primer (5′-CACAAGGCGGTACCCACATCTCTC-3′) were used to PCR amplify a portion of pCR3.1-hERβ corresponding to the 5′ end of hERβ cDNA. The resulting PCR product was substituted into pCR3.1-hERβ via *Nhe*I and *Kpn*I restriction sites. All of the expression vectors that were made using PCR amplification were sequenced to ensure that no errors occurred during their synthesis. The 17mer-E1b-CAT(*ΔTRE*) reporter plasmid was generated by an *Eco*0109-*Hind*III digest of the 17mer-E1b-CAT, which was re-ligated after blunting the restriction ends.

Cell Culture and Transfections

HeLa (human cervical carcinoma) cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Twenty four hours prior to transfections, cells were plated in six-well culture dishes at a density of 3×10^5 cells per well in phenol red-free DMEM with 5% charcoal-stripped fetal bovine serum (sFBS). For transfections, media was replaced with serum-free media and DNA was introduced into cells

in the indicated amounts using Lipofectamine (Invitrogen Life Technologies), according to the manufacturer's guidelines. Five hours later, serum-free media was replaced with phenol red-free DMEM supplemented with 5% sFBS. Twelve hours thereafter, cells were treated with the indicated amounts of various hormones. After 24 hours of hormone treatment (12 hours for inhibitor experiments), cells were harvested and extracts were assayed for chloramphenicol acetyltransferase (CAT) activity, as described previously (47, 48) using butyryl-coenzyme A (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and [^{3}H]chloramphenicol (NEN Life Science Products Inc., Boston, MA). The quantity of resulting radiolabeled product was determined by scintillation counting using biodegradable counting scintillant (Amersham Pharmacia Biotech Inc.) and a Beckman LS 6500 scintillation counter, and then normalized to total cellular protein measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Experiments were done in duplicate and values represent the average ± SEM of at least three individual experiments.

Western Blot Analysis

To determine ER expression levels, cells were transfected as above and harvested. Cell pellets were resuspended in a 50 mM Tris (pH 8.0) buffer containing 5 mM EDTA, 1% Nonidet-P40, 0.2% Sarkosyl, 0.4 M NaCl, 100 μM Na vanadate, 10 mM Na molybdate, and 20 mM NaF and incubated on ice for 1 hour. The lysates were subsequently centrifuged at 21,000 g for 10 minutes at 4 °C. The resulting supernatants were mixed with SDS-PAGE loading buffer, run on a 7.5% SDS-PAGE gel and subsequently transferred to nitrocellulose membrane. The membranes were blocked using 1% nonfat dried milk in 50 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% Tween-20, and sequentially incubated with an anti-Flag M2 antibody (Sigma) and a

horseradish peroxidase-conjugated, anti-mouse antibody. Blots were visualized using enhanced chemiluminescence (ECL) reagents as recommended by the manufacturer (Amersham Pharmacia Biotech Inc.).

RESULTS

Activation of ERα and ERβ by a cAMP signaling pathway

Several studies have demonstrated that agents that stimulate increases in cAMP can promote ERα-dependent gene expression in the apparent absence of hormone (20-23, 33, 49). To determine whether ERβ could be activated in a similar manner, HeLa cells were transiently transfected with expression vectors for either human ERα or ERβ along with an ERE-E1b-CAT synthetic target construct which possesses an estrogen response element (ERE) from the *Xenopus* vitellogenin A2 promoter linked to the adenoviral E1b TATA box and chloramphenicol acetyltransferase (CAT) reporter gene. Cells were subsequently stimulated with either E2 or a combination of forskolin and IBMX (forskolin/IBMX), which increases cAMP production by activating adenylate cyclase and inhibiting phosphodiesterases, respectively, and CAT activity was measured. As expected, E2 stimulated both ERα- and ERβ-dependent transcription of this reporter (**Fig. 1**). Consistent with previous reports (23, 33), stimulation with forskolin/IBMX also resulted in a robust stimulation of ERα transcriptional activity, although a longer (24 h) hormone treatment enhanced the E2-stimulated relative to forskolin/IBMX-stimulated response (compare **Figs. 1** and **2**). Importantly, under the same conditions ERβ was also activated upon stimulation of cells with forskolin/IBMX (**Fig. 1**). Pretreatment with a protein kinase A (PKA)-selective inhibitor, H89 (50), blocked forskolin/IBMX-induced gene expression by both receptor subtypes, thus demonstrating that forskolin/IBMX induction of ERα and ERβ activity is mediated by a cAMP/PKA signaling pathway in these cells. Moreover, this inhibition was

specific for forskolin/IBMX induction, as H89 treatment did not significantly alter basal or E2-stimulated responses for either ERα or ERβ.

An estrogen response element is required for ERα and ERβ activation of ERE-E1b-CAT expression by the cAMP/PKA signaling pathway

To test whether the cAMP signaling response was mediated through an ER genomic mechanism in which the ER binds directly to the promoter, the effects of forskolin/IBMX on ERα and ERβ was assessed on the E1b-CAT reporter, which lacks an ERE. The expected ERα- and ERβ-dependent responses were observed for the ERE-E1b-CAT reporter. As anticipated, basal transcription of the E1b-CAT promoter was minimal and E2 did not further increase CAT gene expression in cells transfected with either ERα or ERβ (**Fig. 2**). Moreover, forskolin/IBMX did not stimulate E1b-CAT reporter gene activity in the presence of transfected ERα, and only a very weak forskolin/IBMX-dependent E1b-CAT expression was observed in the presence of ERβ. These data indicate that ERα- and ERβ-dependent transcription in response to forskolin/IBMX requires the presence of an ERE.

CBP and p160/SRC coactivators enhance cAMP-stimulated ERα and ERβ responses

Estrogen receptor-α-dependent and ERβ-dependent transcriptional differences are partly attributable to the selectivity these receptors possess for the different coactivators in the presence of various ligands (51, 52). In an attempt to extend this concept, we overexpressed the p160 coactivators (SRC-1, TIF2, and RAC3) as well as the general coactivator/cointegrator, CBP, in cells in order to determine whether any of them might distinguish between ERα and ERβ in their ability to potentiate activation by cAMP-dependent signaling. Each of these coactivators

strongly enhanced ERα- and ERβ-dependent transcription in comparison to reporter activity in the absence of exogenous coactivator (**Fig. 3**). However, none of the coactivators selectively enhanced the activity of estrogen-activated receptor over cAMP-activated receptor, nor of one receptor subtype over the other. These results suggest that each of the p160 coactivators and CBP can contribute significantly to the forskolin/IBMX-induced activation of both ERα and ERβ.

Activation of ERα and ERβ by cAMP depends on promoter context

In general, ER-mediated transcription depends on the promoter context in which an ERE is found (10, 53, 54). We therefore tested the extent to which forskolin/IBMX could stimulate ER-dependent gene expression in the context of various synthetic and natural ERE-containing promoters. The pS2 construct contains the -1100 to +10 region of the E2-responsive human pS2 promoter subcloned into a CAT reporter plasmid (43). The pATC0, pATC1, and pATC2 are synthetic reporters that possess 0, 1, or 2 EREs, as indicated in their nomenclature. The ERE-E1b-CAT reporter construct was included in these experiments as a control. There was no E2-induced response for pATC0 since it had no ERE, a weak response for pATC1, and a synergistic E2-dependent response for pATC2, for both ER subtypes (**Figs. 4, A and B**). Notably, there was no forskolin/IBMX-induced response on either of these promoters, demonstrating that the number of EREs, in and of itself, had no effect on the forskolin/IBMX-induced activation. Moreover, while the expected E2-dependent increases were present for both ERα and ERβ on the pS2 promoter, there was no significant increase in reporter activity in response to forskolin/IBMX. Thus, the cAMP/PKA signaling pathway mediates ERα- and ERβ-dependent gene expression in a promoter-dependent fashion.

The upstream TRE enhancer in the ERE-E1b-CAT reporter is required but not sufficient for activation of ERβ-dependent gene activation by forskolin/IBMX

Promoter differences in forskolin/IBMX-induced responses suggested that *cis*-acting factor(s) in addition to EREs contributed to cAMP-stimulated ERα and ERβ transcriptional activities. It has been reported that ERα and ERβ can mediate ligand-dependent responses at TPA response elements (TREs), independent of EREs, when they are tethered to the promoter via AP-1 transcription factor complexes (55, 56). Previous sequence analysis (57) revealed that a putative TRE (TGACACA) that differs from the consensus TRE sequence (TGAGTCA) by two nucleotides resides in the backbone of many plasmid vectors. In ERE-E1b-CAT such a putative TRE is located ~255 base pairs upstream of the ERE. To determine whether this TRE played any role in the ability of forskolin/IBMX to stimulate the activity of either ER, we examined the ability of forskolin/IBMX to stimulate CAT gene expression from a reporter plasmid [ERE-E1b-CAT(mTRE)] in which the TRE was mutated to GGACTCA, a mutation previously demonstrated to abolish AP-1 binding (41, 58). As shown in **Fig. 5A**, the TRE mutation decreased overall reporter gene activity in the presence of both ERα and ERβ whether cells were treated with vehicle, E2, or forskolin/IBMX. However, E2 was still able to increase ERE-E1b-CAT(mTRE) activity above basal for both ERs, and forskolin/IBMX stimulated this reporter's activity in the presence of ERα. In contrast, forskolin/IBMX was unable to stimulate ERβ-dependent transcription of ERE-E1b-CAT(mTRE), suggesting that this putative TRE was necessary for cAMP-induced ERβ-mediated increases in ERE-E1b-CAT reporter gene expression. Similar results were obtained when this TRE was removed by an *Nde*I to *Eco0109*I deletion of a 195 bp region surrounding the site (41) as opposed to mutating it (data not shown).

Western blot analysis indicated that cAMP activation of ERα on the ERE-E1b-CAT reporter (with or without the TRE) was not due to an increase ERα protein levels (**Fig. 5B**). In contrast to ERα, ERβ protein expression is modestly elevated by forskolin/IBMX. However, this does not permit cAMP-stimulated ERE-E1b-CAT(mTRE) activity by ERβ. Thus, cAMP activation of ERβ is dependent on a putative TRE site while loss of this *cis* element only partially attenuates ERα activity and indicates that activation of ERα and ERβ by cAMP signaling are distinct. Taken together with the above data that demonstrated a requirement for an ERE, these data suggest that forskolin/IBMX promotes synergism between either ERα or ERβ and a factor(s) that is bound to a neighboring DNA response element (*i.e.* the putative TRE).

The carboxy terminus of ERα and ERβ mediates forskolin/IBMX-induced transcription, which can be further enhanced by the amino terminus of ERα but not ERβ

Two previous reports have characterized the physical interactions between ERα and the AP-1 transcription factor family member, c-Jun (56, 59). While one of these studies demonstrated that ERα interaction with c-Jun is predominantly mediated by the centrally-located hinge region (domain D) of the receptor (59), both indicated that an interaction with the amino-terminus of ERα is also possible. Therefore, to assess the potential contribution of the amino-terminal A/B domain in mediating forskolin/IBMX-induced responses, ERα and ERβ deletion mutants lacking their A/B domains (ERα-179C and ERβ-143C) were constructed, and the ability of the resulting receptors to stimulate ERE-E1b-CAT reporter activity in response to forskolin/IBMX stimulation was examined. In the absence of transfected ER (**Fig. 6A**, empty), there is very weak CAT gene expression in response to forskolin/IBMX treatment. This minimal promoter activity is substantially less in comparison to activity in the presence of transfected ERs. As shown by

comparison of ERα-179C (ERα amino acids 179-595) with wild type ERα, deletion of the amino-terminus of ERα reduced forskolin/IBMX as well as basal and E2-stimulated activities, which is consistent with this receptor's constitutively active amino-terminal AF-1 domain contributing to E2-dependent ERα responses (7, 10). In contrast, removing the A/B region of ERβ to generate ERβ-143C (ERβ amino acids 143-530) did not reduce forskolin/IBMX induction of ERβ-dependent target gene expression. Notably, the E2-stimulated activity of ERβ-143C is much higher than that of wild type ERβ, which is in agreement with a previously reported inhibitory function for ERβ's amino-terminus (60).

As shown in **Fig. 6B**, ERα but not ERβ retained the ability to stimulate CAT expression from the TRE-minus reporter [ERE-E1b-CAT(mTRE)] in response to forkolin/IBMX (see also Fig. 5). Interestingly, much of the E2-induced and all of the remaining forskolin/IBMX-induced ERα activity is lost when the A/B domain is removed, as shown for ERα-179C, thus supporting the above data that this domain can mediate cAMP-dependent activation of ERα. Taken together, these results demonstrate that domains C thru F of ERα and ERβ are sufficient for cAMP/PKA signaling pathway activation of either receptor provided that an AP-1 DNA binding site is present on the promoter and indicates that this functional interaction is enhanced by the A/B domain of ERα but not ERβ.

Several studies have focused on the ability of the MAPK signaling pathway to stimulate the AF-1 activity of ERα (8, 38). Because it is known that the cAMP/PKA signaling pathway can cross-talk with the MAPK signaling pathway (35), we examined whether the MAPK-directed phosphorylation site in the amino terminus of ERα (Ser^{118}) might be important for the forskolin/IBMX-induced activity of the ERα AF-1 domain. However, mutating this serine to an

alanine (ERα-S118A) did not alter ERα's ability to stimulate transcription of the ERE-E1b-CAT(mTRE) reporter in response to forskolin/IBMX (data not shown). Similarly, alanine mutation of the other known amino-terminal phosphorylated residues in ERα ($Ser^{104/106/118}$ or Ser^{167}) did not inhibit forskolin/IBMX induction of reporter gene expression (data not shown), indicating that these phosphoserine residues did not account for the forskolin/IBMX-dependent activity of the ERα AF-1 domain. Thus, ERα AF-1 activity in response to forskolin/IBMX is likely to be mostly due to cAMP/PKA signaling to a factor(s) that can interact with the A/B domain rather than by altering the phosphorylation of the ERα amino terminus itself.

Functional interactions with the TRE-bound factor(s) can be mediated by the EF region of ERα but not ERβ

It has been demonstrated that amino acids 259 to 302 of ERα constitute a major interaction site with c-Jun (59), and since the ability of ERα-179C to mediate forskolin-induced activation of CAT expression was dependent on a TRE site within the reporter gene that has been shown to support c-Jun physical and functional interactions (41, 57), we investigated the possibility that cAMP activation of ERα-179C was due to a direct interaction between the hinge region of the receptor and c-Jun. To test this hypothesis, the EF domain of ERα (amino acids 302 to 595) and the corresponding ERβ fragment (amino acids 254 to 530) were fused to the Gal4 DNA binding domain (Gal-ERαEF and Gal-ERβEF) and examined for their abilities to stimulate expression of 17mer-E1b-CAT, which contains four Gal4 binding sites upstream of the TATA box and CAT gene. This reporter also possesses the TRE site upstream of the Gal4 binding sites. As expected, both Gal-ERαEF and Gal-ERβEF were stimulated by E2 (**Fig. 7**). Importantly, forskolin/IBMX stimulated ERαEF-dependent expression of the 17mer-E1b-CAT but not the TRE-minus

reporter, 17mer-E1b-CAT(*ΔTRE*). This demonstrates that an ERα construct lacking all known c-Jun binding sites can still be activated and indicates that the interaction between the TRE binding factor and ERα is likely to be indirect and may be mediated via a *trans*-acting factor that can bind to both the EF domain of ERα and a TRE-binding factor such as c-Jun. In contrast, the EF region of ERβ is insufficient to activate reporter gene expression regardless of the presence or absence of a TRE site, indicating that regions within the DNA binding domain and/or hinge of ERβ are important for activation of target gene expression in response to cAMP signaling. Taken together, these results indicate that the EF domains of ERα and ERβ differ in their abilities to mediate ER cooperation with a TRE-bound factor in response to cAMP.

Forskolin/IBMX-stimulated ERα activity is not due to cAMP/PKA-dependent SRC-1 phosphorylation

As mentioned above, mutation of known ERα phosphorylation sites did not inhibit forskolin/IBMX activation of ERE-E1b-CAT(mTRE) by ERα. Interestingly, it had been previously demonstrated that the chicken PR is not phosphorylated in response to cell treatment with 8Br-cAMP but rather cAMP activation of transcription seems to be mediated by an increase in SRC-1 phosphorylation (35). Therefore, we examined whether cAMP/PKA's ability to modulate SRC-1 phosphorylation might also contribute to forskolin/IBMX-dependent activation of ERα. Moreover, SRC-1 binds to both the EF domain of ERα as well as c-Jun (61, 62) and is therefore a good candidate for mediating indirect interactions between these two transcription factors, as described above. Therefore, SRC-1 expression vectors containing substitutions for the two cAMP-induced phosphorylatable residues (T1179/S1185A) or substitutions for all of the seven previously mapped phosphorylation sites [at positions 372, 395, 517, 569, 1033, 1179 and

1185; ref. (63)] were introduced into cells and the abilities of these mutant coactivators to enhance ERα-dependent reporter activity was assessed. Compared to wild type SRC-1 there is an ~20% and ~35% decrease in the ability of the SRC-1$^{T1179/S1185A}$ and the seven-alanine mutant (SRC-1^{7Ala}), respectively, to potentiate forskolin/IBMX-induced ERα activity (**Fig. 8, A and B**). Nonetheless, decreases in ERα coactivation by both the SRC-1$^{T1179/S1185A}$ and the SRC-1^{7Ala} mutants were equal for basal as well as for E2-stimulated and forskolin/IBMX-stimulated responses, suggesting that SRC-1 phosphorylation does not specifically modulate cAMP-dependent activation of ERα.

DISCUSSION

In this report, we demonstrated that forskolin/IBMX, through increased intracellular cAMP and activation of PKA can stimulate ERβ-dependent transcription, as was previously shown to be the case for ERα. However, there are significant differences in the ability of ERα and ERβ to be ligand-independently activated by this mechanism. In particular, a TPA responsive element (TRE) upstream of the ERE was necessary for ERβ-dependent transcription in response to stimulation with forskolin/IBMX, whereas this upstream sequence contributed to, but was not required for activation of full-length ERα. Furthermore, functional interactions with the TRE-bound factor(s) could be mediated by the EF region of ERα but not ERβ. Overexpression of the p160 and CBP coactivators enhanced forskolin/IBMX-induced ERα and ERβ activity, indicating that these coactivators can form functional complexes with the unliganded receptor. However, in contrast to the previously reported importance of SRC-1 phosphorylation for cAMP-mediated activation of chicken PR (35), the contribution of these phosphorylation sites to SRC-1 coactivation of cAMP-induced ERα was minor and not specific to ER activation by

forskolin/IBMX. Taken together, these data demonstrate that the cAMP signaling pathway can stimulate ER-dependent transcription by promoting functional interactions between TRE-bound transcription factor(s), coactivators, and either ERα or ERβ bound to an ERE.

There has been considerable interest in understanding the relative contributions of the ERα and ERβ AF-1 domains in mediating ER-dependent transcription. The A/B domain of ERα, which encompasses the AF-1, is generally more active than that of ERβ (60). Interestingly, while a MAPK signaling pathway can induce ERα and ERβ AF-1 activity in the absence of ligand (8, 9, 38, 64), our results demonstrate that cAMP signaling can stimulate ERα, but not ERβ activity via the AF1 domain. Although it has been reported that the cAMP signaling pathway can stimulate MAPK activity, mutation of the previously identified amino-terminal MAPK phosphorylation site in ERα (8, 38) does not inhibit its activation by forskolin/IBMX. Consistent with this result, previous work has demonstrated cAMP-induced phosphorylation of ERα's carboxy-terminal domain (32); however, the location of these phosphorylation sites *in vivo* remains to be identified. It is therefore likely that forskolin/IBMX activation of ERα via the AF-1 domain is due to cAMP/PKA signaling effects on the recruitment and/or activity of a coactivator(s) that interacts selectively with the AF-1 domain of ERα. Interestingly, there are several coactivators, including the p72/p68 RNA-binding DEAD-box proteins (65, 66) and the RNA coactivator, SRA^2, which have been found to selectively interact with the AF-1 domain of ERα but not that of ERβ. Moreover, in addition to p68 being a phosphorylated protein (67), all three of these coactivators are found in complexes with other coactivator molecules, such as CBP, SRC-1, TIF2, and AIB1 (65, 66), which are known to be phosphoproteins (35, 68-70).

The carboxy-terminal ligand binding domains of ERα and ERβ possess considerably higher sequence homology than do the A/B domains. Interestingly however, there are also differences

in the abilities of the ERα and ERβ EF domains to be activated by cAMP signaling, as indicated by the ability of Gal-ERαEF but not Gal-ERβEF to stimulate 17mer-E1b-CAT activity in response to forskolin/IBMX. Moreover, the inability of the AF-1-deletion mutants (ERα-179C and ERβ-143C) and the Gal-ERαEF chimera to stimulate the activities of reporters lacking TREs [ERE-E1b-Cat(mTRE) and 17mer-E1b-CAT(*ΔTRE*), respectively] in response to forskolin/IBMX suggest that activation of domains EF of ERα and C thru F of ERβ require a functional interaction between these receptor domains and a TRE-bound transcription factor. Based on sequence information, this TRE-binding factor most likely belongs to the AP-1 transcription factor family. This includes Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra1, Fra2), and ATF (ATFa, ATF2, ATF3) proteins, which can form homo/heterodimers among themselves and promote transcription via binding to palindromic sequences [TGA(C/G)TCA] that are found in a number of promoters [reviewed in (71)]. Although the sequence (TGACACA) present in our ERE-E1b-CAT reporter diverges from the consensus TRE sequence, it has been shown to bind c-Jun (58). Moreover, our lab previously demonstrated through over-expression studies that c-Jun can enhance ERα-mediated transcription of ERE-E1b-CAT but not of the corresponding TRE mutant reporter, ERE-E1b-CAT(mTRE) (41). Since we demonstrate a requirement for the ERE and TRE DNA sites, our data suggests that cooperation between ER and AP-1 transcription factors bound to their respective target promoter sequences results in robust forskolin/IBMX activation of target gene expression. Based on the ability of just the EF domains of ERα to be activated by cAMP even though this portion of ERα does not bind c-Jun or c-Fos (59) these interactions between ER and AP-1 transcription factors need not be direct, although we cannot rule out the possibility of other TRE-binding factors directly contacting ERα and/or ERβ.

One potential mechanism through which TRE and ERE binding factors could indirectly interact with one another is through coactivators. We have demonstrated that CBP as well as all three p160 coactivators can enhance forskolin/IBMX-induced transcription of ERE-E1b-CAT by ERα and ERβ, suggesting that these coactivators can form functional complexes with ERs in the absence of hormone. Moreover, overexpression of these coactivators does not compensate for the inability of forskolin/IBMX to stimulate ERβ-dependent transcription of the ERE-E1b-CAT(mTRE) target construct (data not shown), indicating that their ability to stimulate cAMP-induced ERβ function is derived from ERβ and TRE-binding factor interactions. Although the identity of the cofactors critical for interaction between either ER and factors bound to the TRE site have not been identified, there are a number of potential candidates that possess the ability to bind to c-Jun as well as ERs. These include the coactivators SRC-1, JAB1, and CAPER as well as the integrator protein, CBP (61, 72-75). In all cases but CAPER, the coactivator protein utilizes distinct sites to bind to c-Jun and nuclear receptors suggesting that these coactivators are well suited to act as physical bridges between these two classes of transcription factors. While we found that all the p160 coactivators and CBP can contribute to forskolin/IBMX-induced activation of ER target gene expression, suggesting that they may act as factors able to physically and functionally link AP-1 and ER in our model system, we were unable to observe a similar activity by the c-Jun activation binding protein, JAB1 (data not shown).

Cyclic AMP signaling leads to phosphorylation of SRC-1 at Thr^{1179} and Ser^{1185} residues contributing to stabilizing CBP-P/CAF interactions and functional synergy between CBP and SRC-1 (35). Moreover, mutation of these two amino acids to alanines reduced both progesterone-stimulated and, in an even more marked fashion, cAMP-stimulated chicken PR activity in COS cells. However, these mutations did not completely blocked SRC-1's ability to

enhance cAMP-dependent activation of chicken PR, suggesting that phosphorylation of another cofactor(s) may contribute to activation of this receptor by cAMP. The same mutations only slightly impaired the ability of SRC-1 to enhance ERα activity stimulated by E2 and cAMP. Moreover, mutation of all 7 SRC-1 phosphorylation sites identified by Rowan *et al.* (63) also reduced the overall efficacy of this coactivator, but again, regardless of receptor stimulus. These data suggest that SRC-1 is not specifically involved in the activation of ERα by cAMP and that this ligand-independent activity can be mediated by another ERα-interacting cofactor(s). It should be noted that growth factor and protein kinase C signal transduction pathways have been shown to alter the phosphorylation and/or coactivation potential of GRIP1/TIF2, AIB1/RAC3 and p300/CBP coactivators (68-70) and it is possible that cAMP cross-talk with one or more of these factors may be critical for activation of ER transcriptional activity. An examination of this possibility awaits identification of cAMP-induced phosphorylation sites in these coactivators. Taken together, our data indicate that cAMP activation of cPR and ERα differ in the extent to which SRC-1 phosphorylation is required for this process, as well as whether the respective receptors are themselves phosphorylated. In addition, ERα and ERβ differ in their dependence on promoter TRE sites and the minimal region of receptor required to respond to cAMP signaling. Overall, this argues that multiple mechanisms contribute to cAMP activation of nuclear receptor transcriptional activity.

The promoters of endogenous genes typically consist of binding sites for many distinct transcription factors. Importantly, the human pS2 promoter contains binding sites for ERs as well as AP-1 transcription factors (76), indicating that expression of this gene which had previously been demonstrated to be activated by cAMP in an ICI 164,384-inhibited manner might involve cross-talk between ER and AP-1. Unexpectedly, our pS2-CAT reporter was not

activated by ERα nor ERβ in response to forskolin/IBMX treatment. This could be due to cell type differences and/or loss of a promoter region critical for cAMP activation of ER during construction of the pS2-CAT reporter. DNA sequence analyses have enabled us to identify several other target gene promoters containing both TRE and ERE sites. Thus, the ability of the cAMP signaling pathway to stimulate ER-dependent transcription via ER-AP-1 interactions might be applicable to many other ER target genes.

Cooperativity between nuclear receptors and non-nuclear receptor transcription factors has been demonstrated for ERα and other members of the nuclear receptor family. For example, functional interactions between ERα and AP-1 transcription factors have been reported (44), which indicated that the number of AP-1 response elements as well as their distances from the ERE can influence hormone-dependent activity. Moreover, we have previously identified another mode of cross-talk between AP-1 and ERα induced by an agent (SKF 82958) that stimulates AP-1 activity and serves as a weak ligand for ERα (41). In addition, the Ras-MAPK-activated transcription factor, Ets-1, can confer robust ligand-independent activity on vitamin D receptor (VDR), ERα, and peroxisome proliferator activated receptor-α (77). For VDR, this was demonstrated to occur through a direct interaction of the receptor with Ets-1. Interestingly, Ets-1 could restore coactivator interaction with an AF-2-defective VDR/RXR heterodimer, suggesting that Ets-1 might stimulate ligand-independent activity by inducing (or stabilizing) a conformation of VDR that allows interaction(s) with coactivators.

Several models exist whereby nuclear receptor activation (*e.g.*, by GR or RAR) can antagonize rather than cooperate with AP-1 activation at promoters containing the TRE sequence in the absence of the nuclear receptor binding site (74). This is in part explained by the ability of these nuclear receptors to compete for the CBP coactivator, which can interact with both nuclear

receptors and AP-1 transcription factors. Our data not only provides for ER activation by cAMP but suggests an alternative model whereby coactivators can mediate positive functional interactions between ER and AP-1 provided that binding sites for these two factors are present on the same promoter. It is clear that ER-dependent responses cannot be predicted on the presence of an ERE alone but consideration must be given to the complexity of such promoters and how these receptors interact with the various non-receptor transcription factors, either directly or through coactivator/cointegrator molecules. Undoubtedly, the ability of coactivators to integrate responses through various classes of transcription factors adds another level of control and specificity to regulation of gene expression.

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FOOTNOTES

1. The following abbreviations are used: ER, estrogen receptor; PR, progesterone receptor; AF, activation function; DBD, DNA binding domain; ERE, estrogen response element; cAMP, cyclic adenosine monophosphate; 8Br-cAMP, 8-bromo-cAMP; IBMX, 3-isobutyl-1-methylxanthine; SRC-1, steroid receptor coactivator-1; P/CAF, p300/CBP-associated factor; CBP, CREB binding protein; MAPK, mitogen-activate protein kinase; PKA, protein kinase A; H89, N-{2-[p-bromocinnamylamino]-ethyl}-5-isoquinolinesulfonamide; TIF2, transcription intermediary factor-2; RAC3, receptor-associated coactivator-3; AIB1, amplified in breast cancer-1; GRIP1, glucocorticoid receptor-interacting protein-1; AP-1, activated protein-1; TRE, TPA response element; CAT, chloramphenicol acetyltransferase.
2. KMC and CLS unpublished data

FIGURE LEGENDS

FIG. 1: Forskolin and IBMX-induced ERα and ERβ transcriptional activity are dependent upon PKA signaling. HeLa cells were transiently transfected with 10 ng of expression plasmid for ERα ($pCMV_5$-ERα) or ERβ ($pCXN_2$-ERβ) and 1 μg ERE-E1b-CAT reporter plasmid. Cells were subsequently treated for 12 hours with vehicle (0.1% ethanol), 1 nM E2 or 10 μM forskolin + 100 μM IBMX (F/I) following 1 hour pretreatment with either 10 μM H89 (+) or DMSO (-). Values are normalized to the activity of ERα in the absence of hormone and represent the average ± SEM of three independent experiments.

FIG. 2: cAMP/PKA stimulation of ER-dependent transcription requires ER binding to its cognate hormone response element. HeLa cells were transiently transfected with either 10 ng of expression plasmid for ERα ($pCMV_5$-ERα) or ERβ ($pCXN_2$-ERβ) along with 1 μg ERE-E1b-CAT or E1b-CAT. Cells were subsequently treated with vehicle, 1 nM E2, or F/I (10 μM/100 μM) for 24 hours. Values are normalized to ERE-E1b-CAT reporter activity for ERα in the absence of hormone and represent the average ± SEM of three experiments.

FIG. 3: The p160 and CBP coactivators enhance cAMP/PKA-mediated ERα- and ERβ-dependent transcription. HeLa cells were transiently transfected with 10 ng of expression plasmid for ERα ($pCMV_5$-ERα) or ERβ ($pCXN_2$-ERβ) along with 250 ng of expression plasmid for SRC-1e, TIF2, RAC3 or the empty vector (pCR3.1) and 1 μg ERE-E1b-CAT reporter. Cells

were subsequently treated with vehicle, 1 nM E2, or F/I (10 μM/100 μM). CAT measurements were standardized to total protein and results are the averages ± SEM of three experiments.

FIG. 4: cAMP activation of ERα and ERβ depends on promoter context. HeLa cells were transiently transfected with 10 ng of expression plasmid for ERα ($pCMV_5$-ERα) (**A**) or ERβ ($pCXN_2$-ERβ) (**B**) along with 1 μg of the indicated CAT reporter plasmids. Cells were subsequently treated with vehicle, 1 nM E2 or F/I (10 μM/100 μM). Values for ERE-E1b-CAT and pS2-CAT are normalized to their respective vehicle-treated samples, which were arbitrarily set to 100. Values for pATC0, pATC1, and pATC2 are normalized to vehicle treatment for pATC2 which is set to 100. The results are the averages ± SEMs of three experiments.

FIG. 5: A putative AP-1 response element in the target gene promoter is essential for cAMP/PKA-mediated transcription by ERβ. (**A**) HeLa cells were transiently transfected with 10 ng of expression plasmid for ERα ($pCMV_5$-ERα) or ERβ ($pCXN_2$-ERβ) along with 1 μg ERE-E1b-CAT or ERE-E1b-CAT(mTRE). Cells were subsequently treated with vehicle, 1 nM E2, or F/I (10 μM/100 μM). Values are normalized to ERE-E1b-CAT reporter activity for ERα in the absence of hormone and represent the average ± SEM of three experiments. (**B**) HeLa cells were transfected with 1 μg of either Flag-ERα or 3xFlag-ERβ expression plasmid and receptor expression was detected with anti-Flag (M2) antibody. The blot shown is representative of three experiments.

FIG. 6: Different ERα and ERβ domains mediate promoter-specific gene expression in response to cAMP/PKA signaling pathway. HeLa cells were transiently transfected with 10

ng of expression plasmid for ERα (pCR3.1-ERα), ERα-179C (pCR3.1-ERα-179C), ERβ (pCR3.1-Flag-ERβ), ERβ-143C (pCR3.1-ERβ-143C), or empty vector (pCR3.1) along with 1 μg of either (**A**) ERE-E1b-CAT or (**B**) ERE-E1b-CAT(mTRE). Cells were subsequently treated with vehicle, 1 nM 17β-estradiol (E2), or 10 μM forskolin + 100 μM IBMX (F/I). Values are the averages ± SEM of three experiments.

FIG. 7: Mapped c-Jun interaction sites in the ERα A/B domain and hinge are not required for forskolin/IBMX stimulation. HeLa cells were transiently transfected with 100 ng of expression plasmid for Gal-ERαEF (pBind-ERαEF), Gal-ERβEF (pBind-ERβEF), or GAL4 DBD (pBind) along with 1 μg of either 17mer-E1b-CAT or 17mer-E1b-CAT(*ΔTRE*). Cells were subsequently treated with vehicle, 1 nM E2, or F/I (10 μM/100 μM). Values are normalized to Gal-ERαEF activity for 17mer-E1b-CAT in the presence of vehicle and represent the averages ± SEM of three experiments.

FIG. 8: Alanine mutation of SRC-1 phosphorylation sites decreases its coactivation of ERα but is not specific to cAMP/PKA-dependent signaling. (**A**) A representative experiment in which HeLa cells were transiently transfected with 10 ng of expression plasmid for ERα ($pCMV_5$-ERα) along with 1 μg of expression plasmid for either wild type or mutant SRC-1a ($SRC1^{T1179/S1185A}$ or $SRC\text{-}1^{7Ala}$) or the empty vector (pCR3.1) and 1 μg ERE-E1b-CAT reporter. Cells were subsequently treated with vehicle, 1 nM E2, or F/I (10 μM/100 μM). (**B**) Combined results from 7 experiments. Relative coactivation was determined by dividing reporter activity in the presence of wild type SRC-1 by values obtained in the absence of coactivator (pCR3.1) for each treatment group (vehicle, E2, and F/I) and defining this value as 100. Values for mutant

SRC-1's coactivation are given relative to wild type SRC-1 coactivation. Results are the averages ± SEM of 7 experiments.

Figure 1

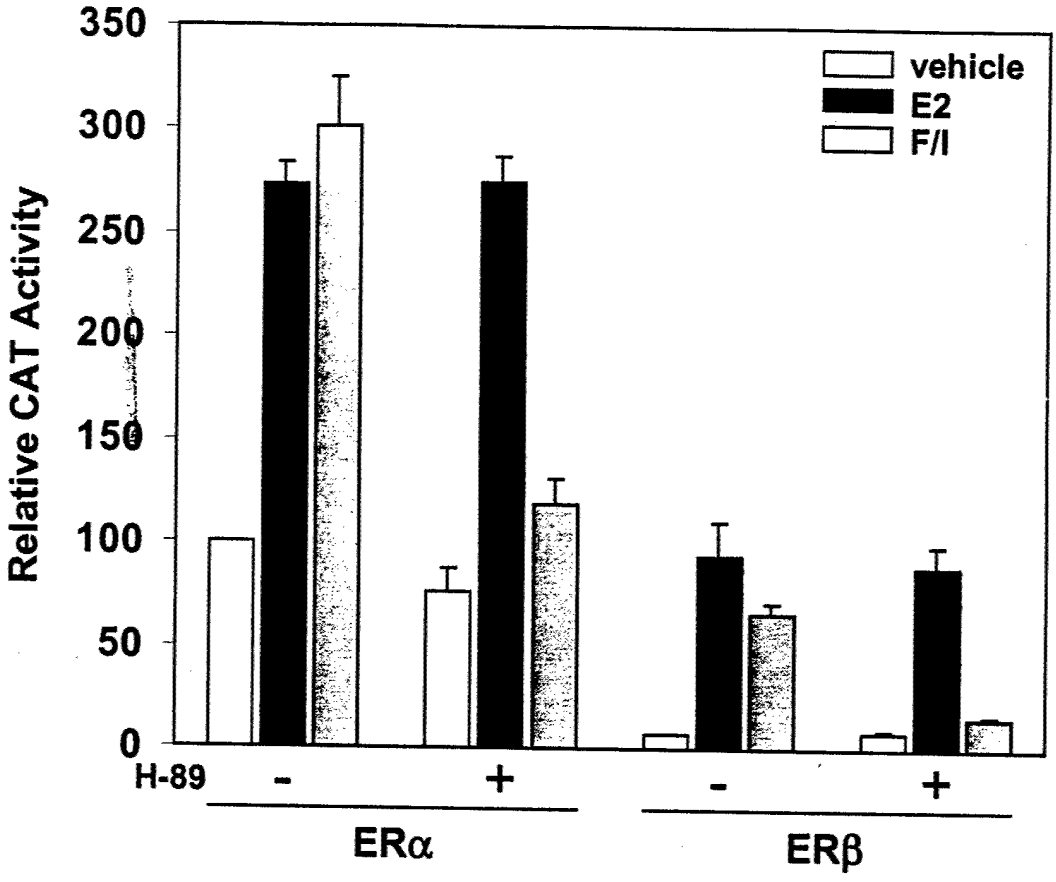

350
300
250
200
150
100
50
0
Relative CAT Activity
vehicle
E2
F/I
H-89
-
+
-
+
ERα
ERβ

Figure 2

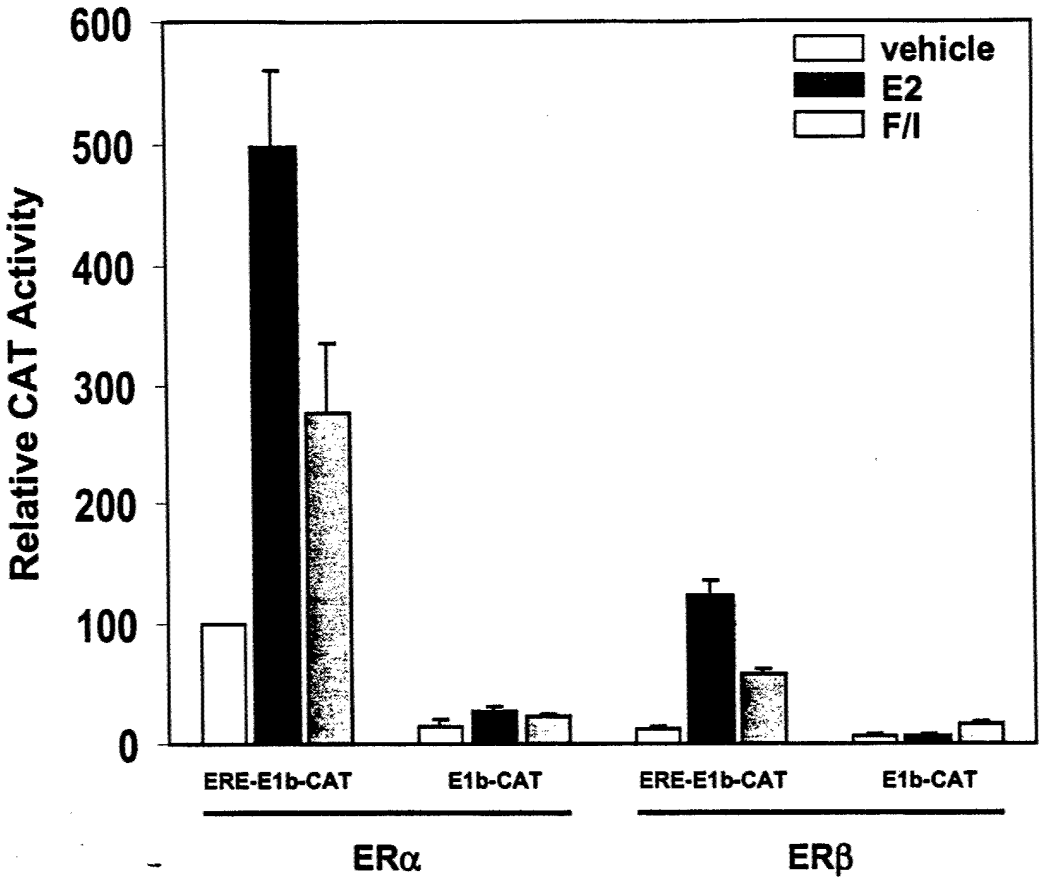

600
500
400
300
200
100
0
Relative CAT Activity
vehicle
E2
F/I
ERE-E1b-CAT
E1b-CAT
ERE-E1b-CAT
E1b-CAT
ERα
ERβ

Figure 3

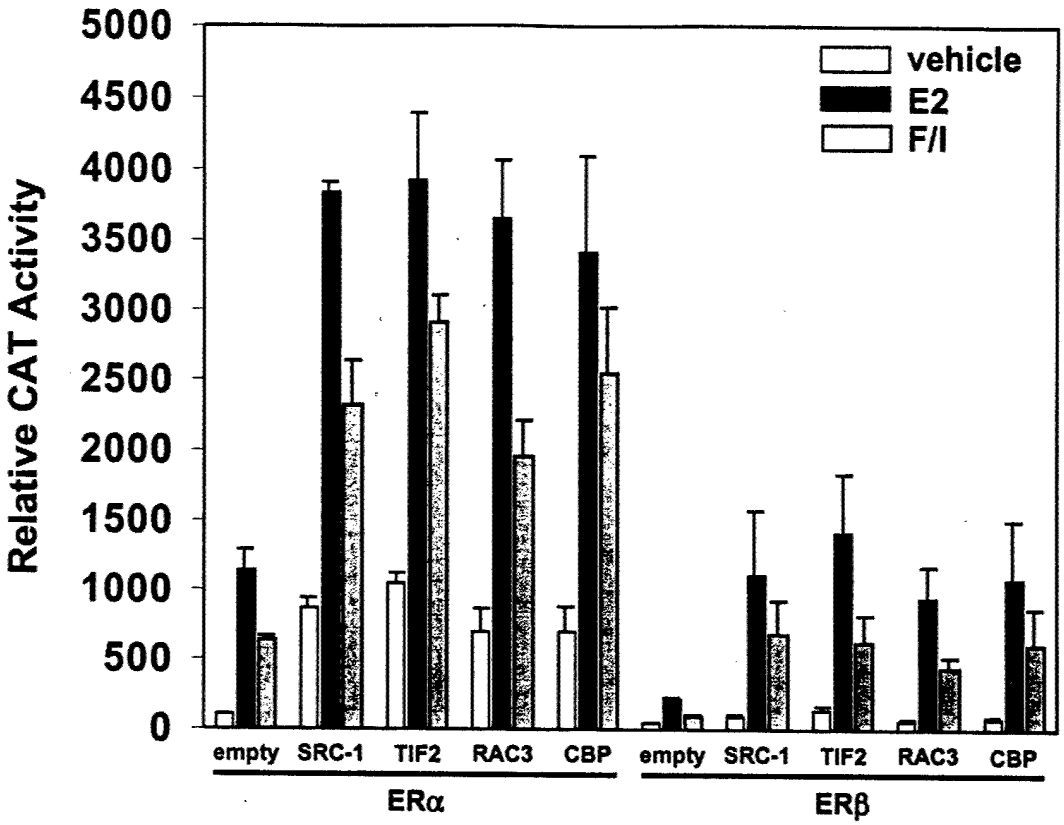

Relative CAT Activity
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
vehicle
E2
F/I
empty
SRC-1
TIF2
RAC3
CBP
empty
SRC-1
TIF2
RAC3
CBP
ERα
ERβ

Figure 4

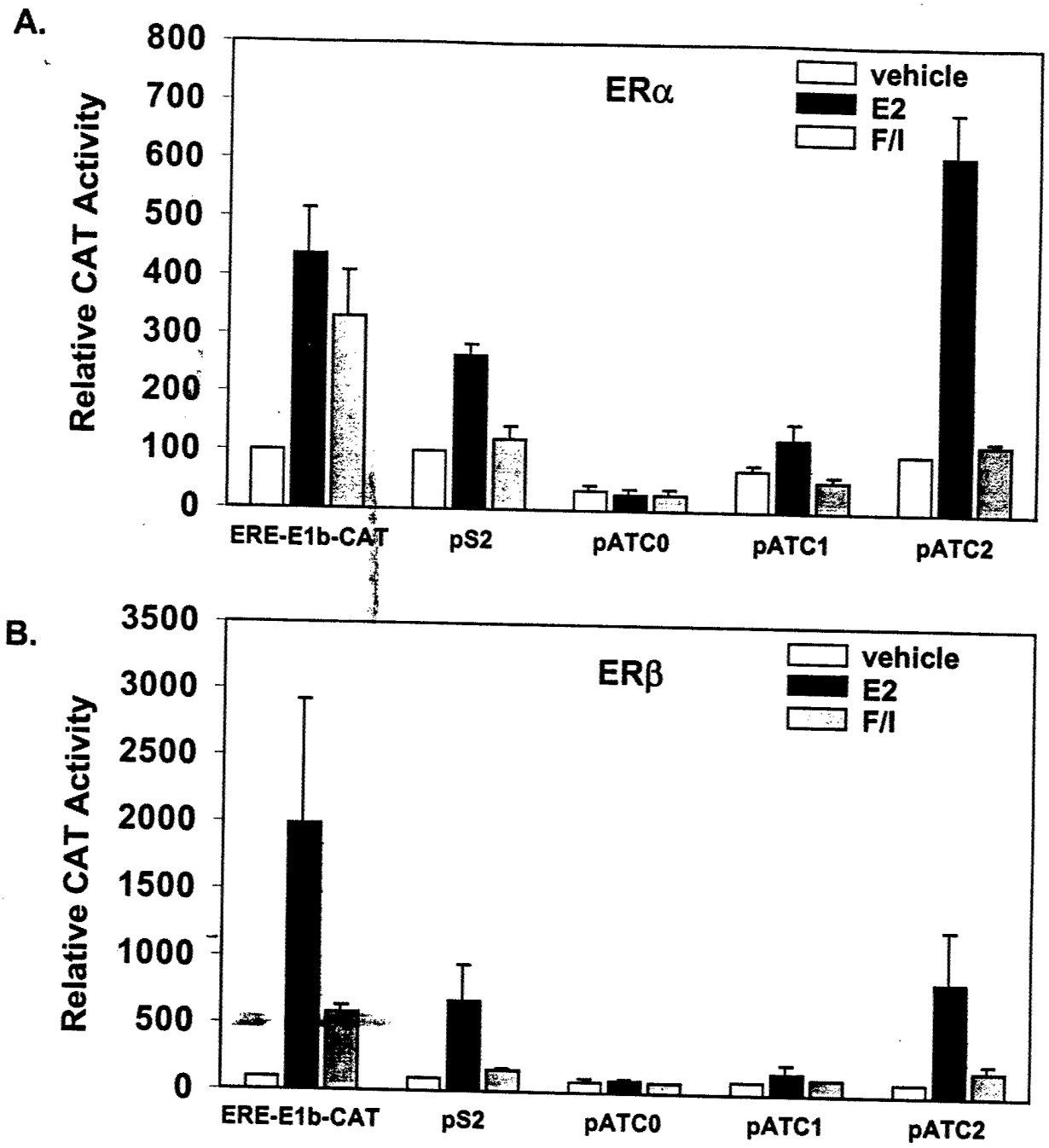
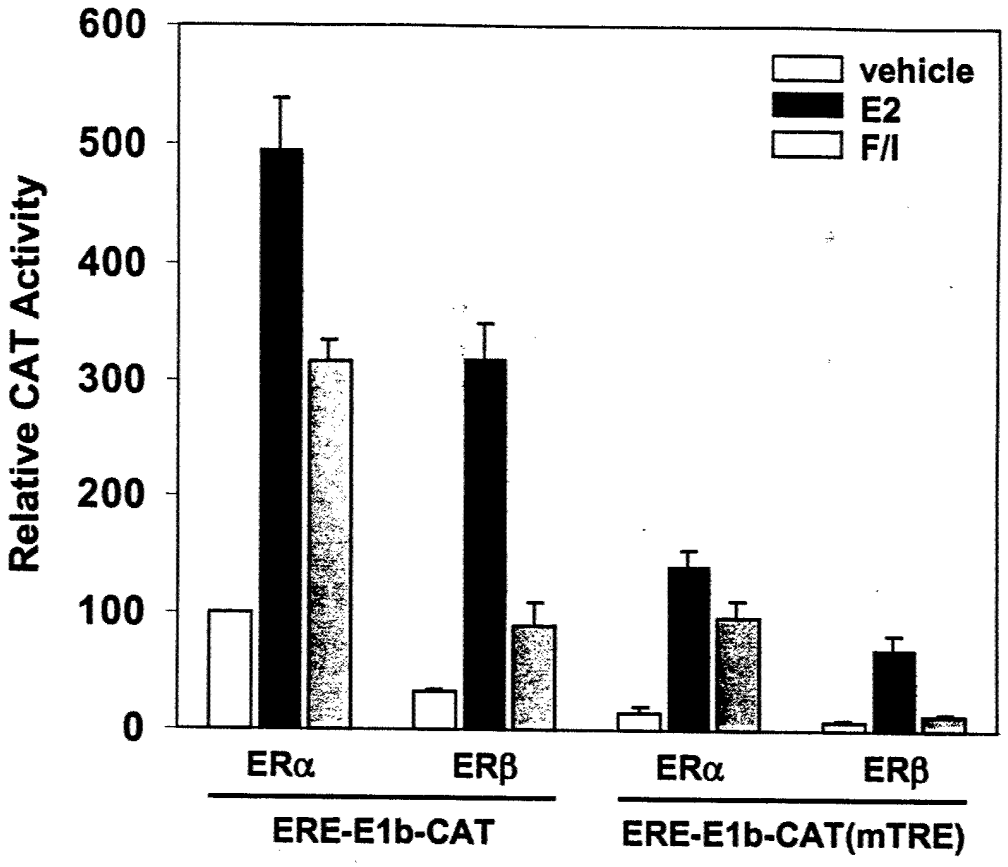

A.
Relative CAT Activity
800
700
600
500
400
300
200
100
0
ERα
vehicle
E2
F/I
ERE-E1b-CAT
pS2
pATC0
pATC1
pATC2
B.
Relative CAT Activity
3500
3000
2500
2000
1500
1000
500
0
ERβ
vehicle
E2
F/I
ERE-E1b-CAT
pS2
pATC0
pATC1
pATC2

Figure 5

A.


600
500
400
300
200
100
0
Relative CAT Activity
vehicle
E2
F/I
ERα
ERβ
ERα
ERβ
ERE-E1b-CAT
ERE-E1b-CAT(mTRE)

B.

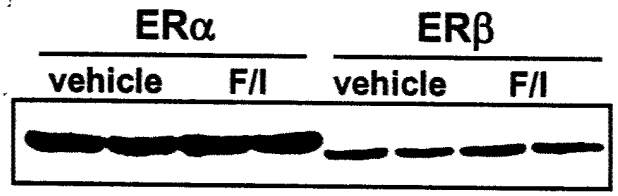

ERα
ERβ
vehicle
F/I
vehicle
F/I

Figure 6

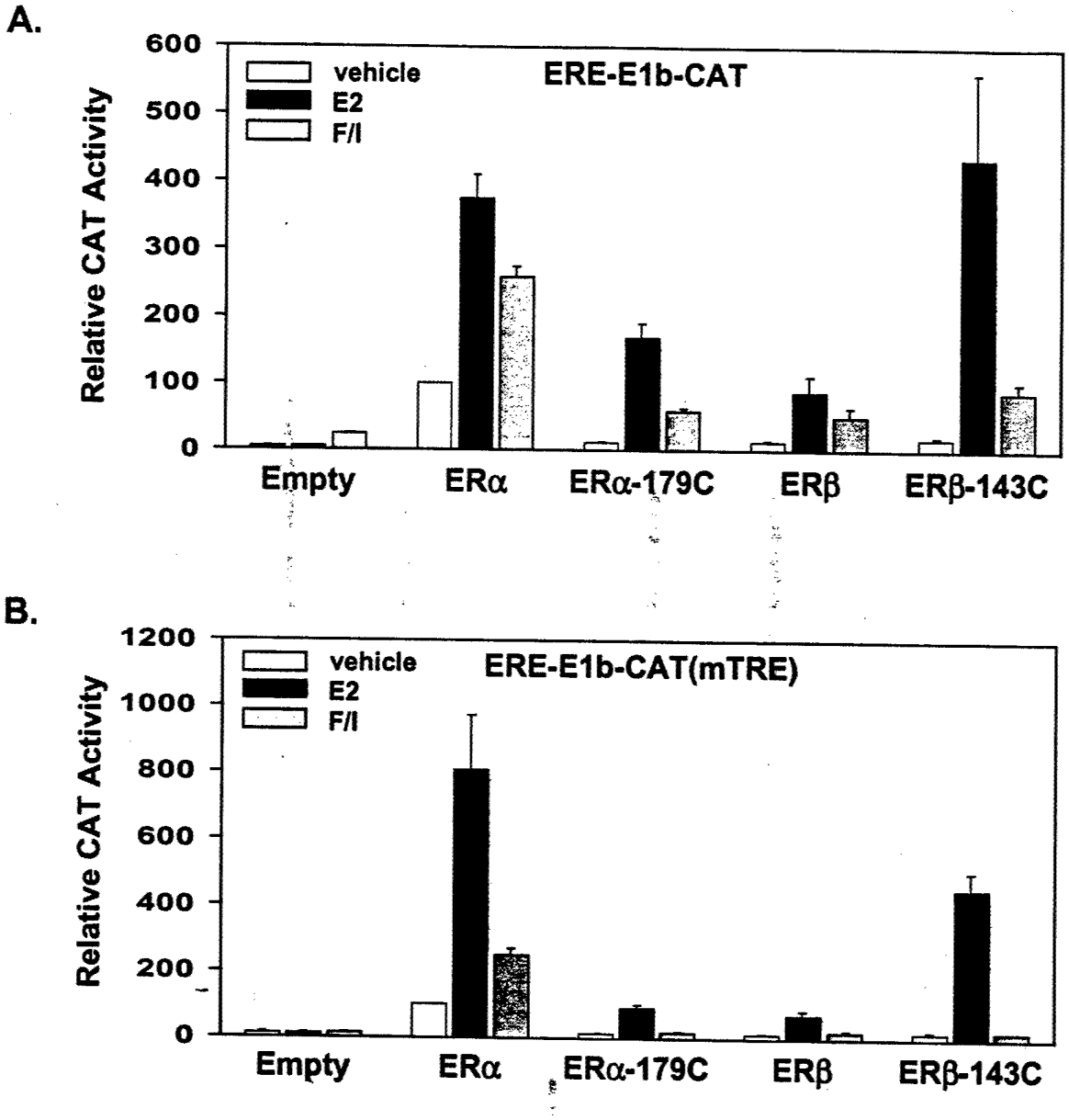

A.
Relative CAT Activity
600
500
400
300
200
100
0
vehicle
E2
F/I
ERE-E1b-CAT
Empty
ERα
ERα-179C
ERβ
ERβ-143C
B.
Relative CAT Activity
1200
1000
800
600
400
200
0
vehicle
E2
F/I
ERE-E1b-CAT(mTRE)
Empty
ERα
ERα-179C
ERβ
ERβ-143C

Figure 7

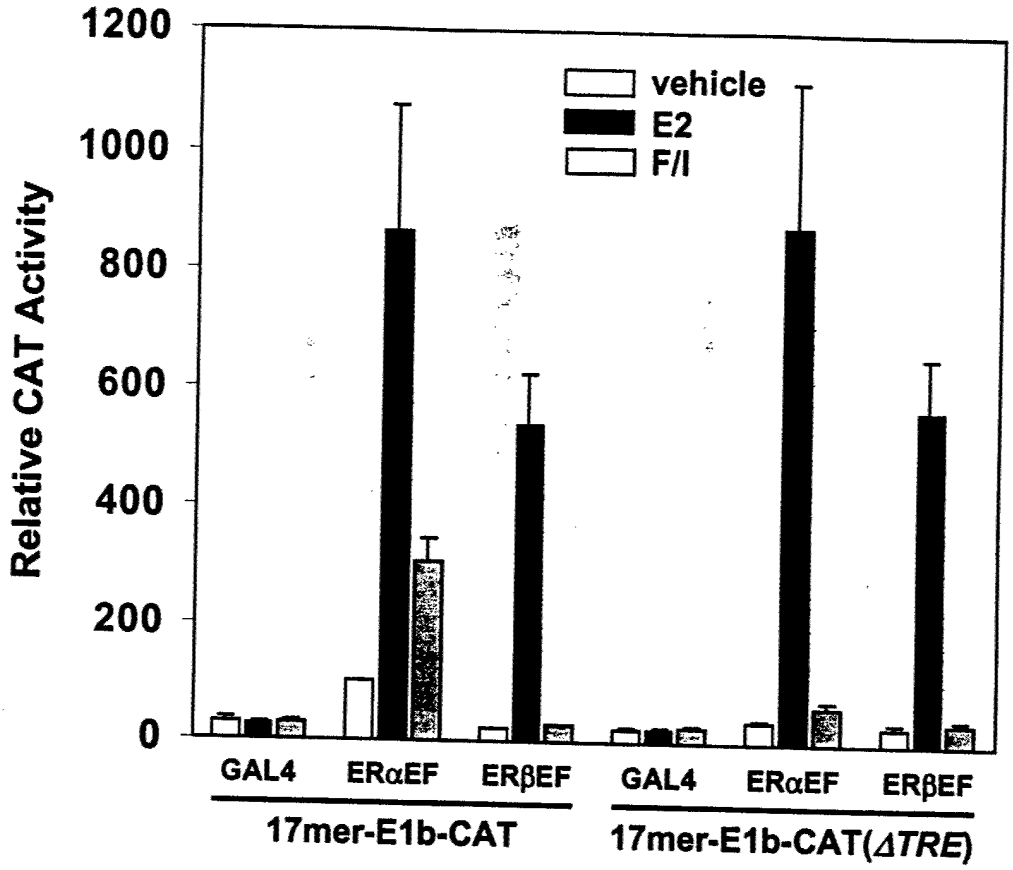
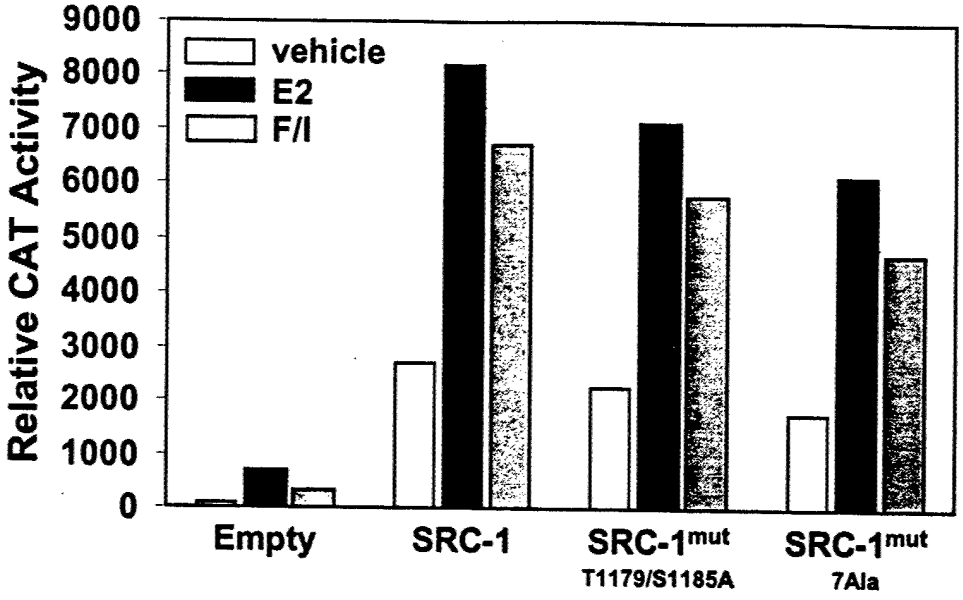
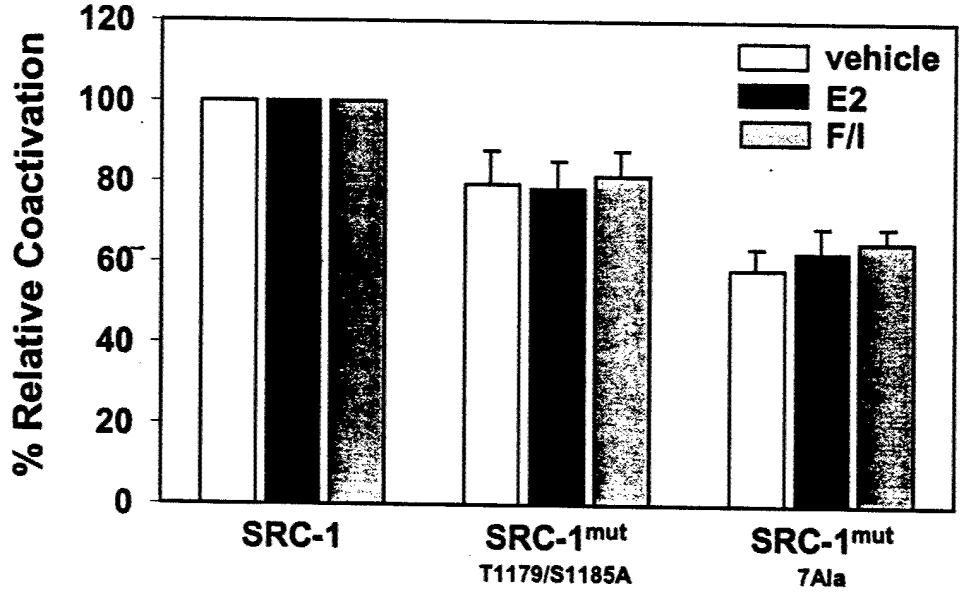

1200
1000
800
600
400
200
0
Relative CAT Activity
vehicle
E2
F/I
GAL4
ERαEF
ERβEF
GAL4
ERαEF
ERβEF
17mer-E1b-CAT
17mer-E1b-CAT(ΔTRE)

Figure 8


Relative CAT Activity
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
vehicle
E2
F/I
Empty
SRC-1
SRC-1mut
T1179/S1185A
SRC-1mut
7Ala


% Relative Coactivation
120
100
80
60
40
20
0
vehicle
E2
F/I
SRC-1
SRC-1mut
T1179/S1185A
SRC-1mut
7Ala

Effects of Phosphorylation Site Mutations in ERα and SRC-1 on Basal, Estradiol- and Cyclic AMP-induced ERα Activity.

Martin Dutertre, Kevin M. Coleman, Brian G. Rowan, Nancy L. Weigel and Carolyn L. Smith. Molecular & Cellular Biology, Baylor College of Medicine, Houston, TX 77030.

Estradiol (E2) increases ERα transcriptional activity in part by enhancing the interactions of its C-terminal ligand-binding domain (LBD) with the p160 (SRC-1, TIF2 and RAC3) and CBP coactivators. We show that these cofactors can also interact with the full-length ERα in a ligand-independent manner *in vivo* and enhance both basal and cyclic AMP (cAMP)-induced activity of the receptor. Since ERα and SRC-1 activity are regulated by specific phosphorylation sites (P-sites), we compared the role of these sites in ERα activity and coactivation following different stimulations. Ala mutation of the Ser^{118} and $Ser^{104/106/118}$ P-sites in the N-terminal ERα-A/B region markedly decreased both basal and E2-induced ERα activity, whereas Ala mutation of the ERα Ser^{167} P-site only sightly affected basal activity. In contrast, only the S104/106/118A mutations modulated cAMP-induced activity. The latter mutations decreased ERα coactivation by p160s and CBP to a much greater extent in basal conditions than under E2 or cAMP stimulation, whereas the S167A mutation augmented basal and cAMP-induced coactivation by p160s. In basal conditions, both the S104/106A and S118A mutations decreased SRC-1 action by two mechanisms: an effect on SRC-1 coactivation that did not require ERα domains outside the A/B; and a seemingly indirect effect on SRC-1 recruitment that did. The latter finding was consistent with the S104/106/118A mutations affecting SRC-1 enhancement of ligand-independent A/B-LBD physical interaction. Finally, combined mutation of all seven known P-sites in SRC-1 ($Ser^{569,395,1033,372,517,1185}$ and Thr^{1179}) decreased its coactivation of ERα activity either basal, E2- or cAMP-induced, by ~40%. Altogether, our data help understand the mechanisms by which ERα-coactivator interactions are regulated by intracellular signaling pathways.

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Meeting: D4 – Nuclear Receptor Superfamily
Poster Session 1: Nuclear Receptor and cofactors, Structure/Function and Transcription

Activation of ERα and ERβ by cAMP Signaling Pathway: Mechanistic Differences and SRC Coactivator Contributions

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Estrogen receptor-α (ERα) can be activated by a cAMP/protein kinase A signaling pathway in the apparent absence of hormone. We report that this signaling pathway can also stimulate ERβ-dependent transcription of an estrogen response element (ERE)-containing reporter gene (ERE-E1b-CAT) in HeLa cells in response to treatment with forskolin plus 3-isobutyl-1-methylxanthine (F/I); agents which increase intracellular cAMP. However, cAMP-induced ERβ-dependent gene expression required an upstream TPA response element (TRE) in addition to the ERE, whereas F/I induction of ERα transcriptional activity was observed in target genes lacking the upstream enhancer. All three p160 coactivators (SRC-1, TIF2 and RAC3) as well as CBP stimulated F/I-induced ERα and ERβ activity indicating that they can form functional complexes with both ERs in the absence of exogenous ligand. While these coactivators also increased ERα transcriptional activity induced by either estradiol (E2) or F/I on a target gene lacking a TRE, they stimulated only E2-induced ERβ activity. This indicates that coactivator overexpression was unable to compensate for lack of a TRE with respect to cAMP activation of ERβ, and suggests that ERβ/AP-1 interactions are required for this response. Phosphorylation of SRC-1 has been previously shown to contribute to chicken progesterone receptor-dependent transcription stimulated by 8Br-cAMP and the role of SRC-1 phosphorylation in F/I activation of ERα activity was therefore assessed. Mutation of the two cAMP-induced SRC-1 phosphorylation sites (T1179A/S1185A) modestly impaired this coactivator's ability to enhance ERα-mediated gene expression under basal, E2 or F/I treatment conditions, indicating that cAMP-dependent phosphorylation of SRC-1 does not contribute specifically to F/I-mediated activation of ERα. Taken together, this data highlights mechanistic differences in activation of PR, ERα and ERβ by cAMP signaling pathways.

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Meeting: D4 – Nuclear Receptor Superfamily
Poster Session 1: Nuclear Receptor and cofactors, Structure/Function and Transcription